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Immunomodulatory potential of different herbal plant extracts on striped catfish (Pangasianodon hypophthalmus) in vitro and in vivo approaches

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A dissertation submitted by
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By Truong Quynh Nhu

ABSTRACT

Striped catfish (*Pangasianodon hypophthalmus*) is one of the economically important fish species, which is widely developed in several Asian countries. Like other fish species, striped catfish also suffer from many kinds of diseases including bacteria, fungi and parasites under stress conditions due to over-expansion farming areas and increase intensification. To control the diseases, regular use of antibiotics and other chemotherapeutics has been causing many negative impacts on the environment and food safety. Therefore, there is an increased demand to look for eco-friendly preventive measures to promote sustainable aquaculture. In recent years, the application of medicinal plants has attracted a lot of attention and become the active subject of scientific investigation. The present study aimed to elucidate the efficiency of different plant extracts in striped catfish in order to find an alternative replacement and reduce the use of antibiotics.

Many medicinal plants have been documented to possess positive effects on the immune responses of aquatic cultivated animals. However, the use of natural products has not been yet popularly applied in striped catfish farms. It may spend a lot of time and the number of experimental animals when all plant products were examined in case of an *in vivo* application. Hence, based on bibliography review data and on a survey in fish farms of Mekong Delta, 20 plants possessing potential immunostimulatory activities were selected for *in vitro* study using the striped catfish peripheral blood mononuclear cells -PBMCs and head kidney leukocytes-HKLs models. Five plant extracts were then validated for their effects on the blood indices, humoral immune responses and disease resistance in striped catfish via oral administration. Moreover, the effects of single supply diets versus mixture diets with *Phyllanthus amarus* and *Psidium guajava* extracts on striped catfish health were continuously submitted. In addition, the protein expression profile was also investigated to provide a better understanding of the metabolic pathways related to immune response, antioxidation and lipid metabolism in fish liver after oral administrated plant extracts. The different polarities of the extract solvents may cause a wide variation in the level of bioactive compounds in the extract, which may lead to different effective degrees on aquatic animal immune responses. The immunomodulatory effects of crude ethanol extract of *P. amarus* and *P. guajava* as well as their fractions including n-hexane, dichloromethane, ethyl acetate, aqueous and non-tannin were therefore compared via *in vitro* experiment. The effects of different pure compounds isolated from *P. amarus* and *P. guajava* were also investigated in this study. Furthermore, biological activities plant

products related to inflammation are largely unknown in fish. The molecular mechanism behind the effects of *P. guajava* extracts on fish immune responses were also highlighted and discussed.

The results demonstrated the ability of several plant extracts in activating of humoral immune responses (lysozyme, complement, and total immunoglobulin) in a dose dependent manner in striped catfish PBMCs and HKLs after 24 h. Several extracts also induced a strong upregulation of 4 cytokines (*il1β*, *ifnγ 2a* and *2b*, and *a2 mhc class II*) according to the concentration, time points and kind of leukocytes. Of the ethanol extracts, *Phyllanthus amarus*, *Psidium guajava*, *Mimosa pudica*, *Azadirachta indica* and *Euphorbia hirta* could stimulate the striped catfish innate immune response (lysozyme and complement) and adaptive immune response (total Immunoglobilin) as well as protecting better resistant capacity in striped catfish against *Edwardsiella ictaluri* pathogen. The study was further revealed how single or mixture supply of *P. amarus* and *P. guajava* extract-based diets acted in the regulation of immune responses, significantly reducing fish mortality. Moreover, oral administration of *P. amarus* and *P. guajava* extracts displayed an upregulation of several proteins involved in immune response in striped catfish liver, the positive synergistic effects of liver proteome profile related to immune system processes were observed under mixture of administration. The crude ethanol extracts of *P. guajava* and *P. amarus*, their fractions and the pure compounds at certain concentration can potentially act as immunomodulators in HKLs of striped catfish at 24h. *P. guajava* dichloromethane fraction beneficially enhanced TLRs-MyD88-NF-κB signaling pathway, following induce the levels of inflammatory and apoptosis cytokines as well as lysozyme, iNOS and RBA productions, providing the better understanding about the mechanism behind the effects of *P. guajava* extracts on fish immune response at molecular level. Taken together, our studies suggested that boosting the immune response by ecologically friendly products, especially *Phyllanthus amarus* and *Psidium guajava* derived products is an effective strategy to promote sustainable aquaculture via improvement the health status as well as resistance to pathogens in striped catfish.

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List of abbreviations

PBMCs	Peripheral blood mononuclear cells
HKLs	Head kidney leukocytes
DMSO	Dimethyl sulfoxide
PMSF	Phenylmethylsulfonyl fluoride
FBS	Fetal bovine serum
RRBC	Rabbit red blood cell
NBT	Nitroblue tetrazolium
il1β	Interleukin 1 beta
ifnγ	Interferon gamma
mhc class II	Major histocompatibility complex class II
ROS	Reactive oxygen species
NOS	Nitric oxide synthase
TLRs	Toll-like receptors
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer
RBA	Respiratory burst activity
FAO	Food and Agriculture organization
BNP	Bacillary Necrosis Pangasius
MAS	Motile aeromonad septicemia
CFU	Colony-forming unit
MOS	Mannan oligosaccharide
XOS	Xylooligosaccharide
GOS	Galactooligosaccharide
FOS	Fructooligosaccharide
FCR	Fish conversion rate
LPS	*Escherichia coli* lipopolysaccharide
RBC	Red blood cell
WBC	White blood cell
ISKNV	Infectious Spleen and kidney Necrosis Virus
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
ACH50	Alternative complement haemolysis
Ig	Immunoglobulin
*cox*2	Cyclooxygenase
inos	Inducible nitric oxide synthase
il8	Interleukin 8
tnfα	Tumor necrosis factor alpha
ifnα	Interferon-alpha
il12	Interleukin 12
tgfβ1	Transforming growth factor beta 1
il4/*13a*	Interleukin *4*/*13a*
il17d	Interleukin *17d*
il10	Interleukin *10*

traf6	Tumor necrosis factor receptor associated factor 6
tp53	Tumor protein 53
CAT	Catalase
SGR	Specific growth rate
WG	Weight gain
CF	Condition factor
MPO	Myeloid peroxidase
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
DEPs	Differentially expressed proteins
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NLRC3	NLR family CARD domain-containing protein 3
CASP8	Caspase-8
CASP3	Caspase-3
ZNF501	Zinc finger protein 501
ERLIN1	Erlin-1
KRT18	Keratin-type I cytoskeletal 18
HMGXB4	HMG domain-containing protein 4
PRDX3	Peroxiredoxin-3
TUBB	Tubulin beta chain
NLRP12	NACHT, LRR and PYD domains-containing protein 12
NLRP3	NACHT, LRR and PYD domains-containing protein 3
RAG1	Recombination activating protein 1
CD8B	T-cell surface glycoprotein CD8 beta chain
HSP90AA1	Heat shock protein HSP 90-alpha 1
HSP90AB1	Heat shock protein HSP 90-beta
PDIA3	Protein disulfide-isomerase A3
TUBA1C	Tubulin alpha-1C chain
CCKAR	Cholecystokinin receptor type A
GNAS	Guanine nucleotide-binding protein G(s) subunit alpha
GRIN2D	Guanine nucleotide-binding protein G(s) subunit alpha
GRIN2D	Glutamate receptor ionotropic NMDA 2D
PLCG1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1
PRKCA	Protein kinase C alpha type
SLC25A5	ADP/ATP translocase 2
VDAC2	Voltage-dependent anion-selective channel protein 2
ACTN4	Alpha-actinin-4
GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2
LCK	Tyrosine-protein kinase Lck
CARD9	Caspase recruitment domain-containing protein 9
ALDH9A1	4-trimethylaminobutyraldehyde dehydrogenase
ACADL	Long-chain specific acyl-CoA dehydrogenase
ADH5	Alcohol dehydrogenase 5 class-3

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SECTION I

Chapter 1

General introduction

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1. Striped catfish production

Striped catfish (*Pangasianodon hypophthalmus*) is one of the commercially important fish in the Mekong River, as well as in Southeast Asia (Roberts and Vidthayanon, 1991, Rainboth, 1996b). However, the increasing trend of striped catfish production was reported over the years. The annual capture of striped catfish production was over 400,000 tons during 1994 and 1999 in Cambodia, which could contribute to 10-15% to the total annual capture fisheries (So and Nao, 1999). FAO (2019) reported that the productivity of striped catfish in Bangladesh, India, Nepal, Singapore, Thailand reached 420,000 tons, valued at 470,000 USD in 2008 (Fao, 2019). Moreover, the global production and value were steadily increasing more than double and reached the highest peak at about 1 million tons and 1,450,000 USD in 2017, respectively (Fig. 1).

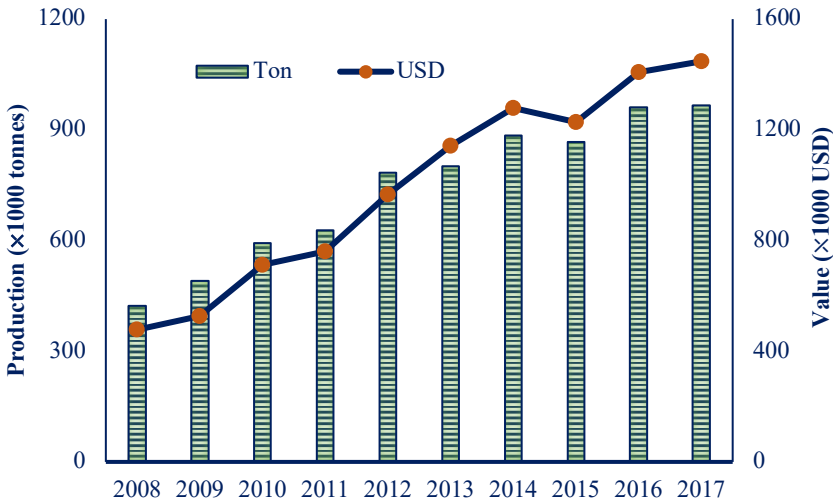

Figure 1. The global production and gross value of cultured striped catfish (FAO, 2019).

Vietnam owns the largest producer of striped catfish since artificial insemination was successfully developed in the mid-1990s (Bui *et al.*, 2010). From this time, the striped catfish industry increased in terms of production tonnage, as well as the culturing area. The statistics showed that the total striped catfish production in Mekong Delta was increased from 683,000 tons in 2007 to 1.25 million tons in 2008 (Economics and Vietnam, 2009). Comparing to total aquaculture production over the years, the increase of striped catfish farming was accounted for 30%, which become the most important species in the Vietnam aquaculture industry (Phan *et al.*, 2009). Striped catfish were also seen as an alternative to white fish and their frozen fillets could be exported to over 100 countries (Phan *et al.*, 2009). It is reported that the export value of striped catfish in 2007 was nearly 645 million US$ and 700 million US$ in the first seven months of 2008 (Economics and Vietnam, 2009). Moreover, the striped catfish production in Vietnam was continuously increased and reached its highest peak at 1.42 million tons, and the value was 2,260,000 USD in 2018 (Mard, 2018). It could be concluded that the striped catfish culture in Mekong Delta has spectacularly developed in over the past decade, which is considered as a success story of aquaculture in Viet Nam (Phuong and Oanh, 2009, De Silva and Phuong, 2011). Over the last decade there has been three farming systems operated including cage, pond and pen, and pond culture has become predominant and currently this form dominates striped catfish farming in the Delta. The farm size and the water surface area ranged from 0.2 to 30 ha and 0.12 to 20 ha, respectively. The number of ponds per farm and pond size ranged from 1 to 17 and 0.08 to 2.2 ha, respectively. The pond depth in striped catfish farms ranged from 2.0 to 6.0 m with the great majority of farms (69%) with pond water depths of 3.5 to 4.5 m (Phan *et al.*, 2009). The striped catfish farming system produced on average over 200–400 ton/ha/crop which occupied approximately 6000–7000 ha of land (De Silva and Phuong, 2011).

2. Habitat and biology

Striped catfish is called sutchi catfish, and is commonly known as a unique tropical freshwater fish native to the rivers of Southeast Asia. The striped catfish is a long-distance migration catfish. They refer to inhabit the large tropical river such as the Mekong river system and Chao Phraya basins (Pritchard *et al.*, 2000). Striped catfish start to spawn in Laos (upstream) from May to July and then swim to the delta region (downstream) between September and December (Li *et al.*, 2013). This aquatic animal has been widely cultured in Vietnam, and, to a lesser extent, in Thailand, Malaysia, Indonesia, Laos, Cambodia, and China (Roberts and Vidthayanon, 1991, Subagja *et al.*, 1999, Chheng *et al.*, 2004, Ali *et al.*, 2005, Amin, 2005). This species has been widely cultured in Thailand for more than 50 years by using the naturally domesticated stocks from the Chao Phraya river (Boonbrahm *et al.*, 1967, Sidthimonk and Pinyoying, 1968). In recent years, the striped catfish was successfully introduced and popularized to culture in India and Bangladesh (Singh and Lakra, 2012). In Vietnam, the striped catfish farms mainly distributed in Dong Thap, Can Tho, and An Giang province (Phan *et al.*, 2009).

Among the *Pangasiidae* family, striped catfish is a larger-sized species distributed in the Mekong Delta (Rainboth, 1996a, Kottelat, 2001), it can reach a total length of 130 cm

(Lorenzen *et al.*, 2006) and 44 kg in weight. The mature fish reach an age of three years in nature or more in captivity, with minimal weight and length of 4 kg and 54 cm, respectively (Van Zalinge *et al.*, 2002). In captivity conditions, striped catfish reach the harvest size of 1.0 to 1.5 kg after a growth period of about 5 to 6 months depending on the size of the fingerlings stocked.Striped catfish is an omnivorous species that could eat plants or fruits (vegetable debris) (Rainboth, 1996b).

Under dark conditions, the fish larvae reduced their cannibalism capacity and the survival rates was increased (Mukai, 2011). The fish could be tolerant to the high stocking density of 150 fish/m^3 in artificial conditions (Islam *et al.*, 2006). The striped catfish is a widely tolerant species, which could adapt in the pH ranging between 6.5 and 7.5, and the temperature from 22 to 26 °C. However, Phuc *et al.* (2017) reported that the striped catfish survival rate was more than 90% in the rearing condition of until 35 °C and 6‰ salinity. Striped catfish maintain a standard metabolic rate through aquatic breathing alone in normoxia, but their air-breathing is important in the case of hypoxia (Lefevre *et al.*, 2011a). Lefevre *et al.* also mentioned that the striped catfish could survive under lower level of oxygen at 2 kPa and is highly tolerant to nitrite up to 1.95 mM after 96h (Lefevre *et al.*, 2011a, Lefevre *et al.*, 2011b). Moreover, metabolic as well as immune pathways are enhanced in striped catfish head kidney under 21-day of salinity stress condition up to 10 ppt (Schmitz *et al.*, 2017).

3. Disease in striped catfish populations

In recent years, to maximize profit, the farmer tried to intensify the farming area and fish density to increase production. The intensive production of striped catfish in open farming systems has been generally accompanied by the outbreak of infectious diseases. Several studies documented that parasites, bacteria, and fungi were the primary pathogens that mostly caused mortality in striped catfish farms (Dung *et al.*, 2008, Ly *et al.*, 2009, Székely *et al.*, 2009, Baska *et al.*, 2009a, Tien *et al.*, 2012, Duc *et al.*, 2015). The prevalence and intensity of parasite infections usually occur in early larval stages due to high stocking density, water temperature variation and organic load in culture ponds. Parasite infections in striped catfish are mainly external parasite infections (i.e. *Trichodina* spp., *Apiosoma* or *Dactylogyrus* spp., or *Gyrodactylus* spp), and internal parasites (i.e. *Myxobolus* spp., *Henneguya* spp., *Ichthyonyctus pangasia*, *Cucullanellus minutus* and *Bucephalosis gracilescens*). Among the infectious parasites, the prevalence of monogenean (*Dactylogyrus* sp.) infestation was about 60 to 90% in striped catfish farms (Lakra and Singh, 2010, Singh and Lakra, 2012). The gill fluke usually causes high mortality in striped catfish during the first week of stocking (Lakra and Singh, 2010). Moreover, striped catfish infected with different densities of monogenean *Thaparocleidus* sp. could modulate the innate immune response, as well as oxidative stress biomarkers (Kumar *et al.*, 2017). Thuy *et al.* (2010) also showed that trematode metacercariae including *Haplorchis pumilio*, *H. taichui*, *Centrocestus formosanus*, and *Procerovum* sp. were detected in the body musculature, head, fins and especially at the base of striped catfish fins. The prevalence of trematode metacercariae in fish was higher in the rainy season compared to the dry season. However, fish at the age of 61 to 90 days post-hatch present the highest intensity and prevalence of those metacercariae, up to 10.58 and 26.98%, respectively. Additionally,

recent documents also highlighted the presence of new myxosporean species (i.e. *Myxobolus omari* sp. nov. and *M. leptobarbi* sp. nov.) from striped catfish pond farms and net-cages, (Székely *et al.*, 2009, Baska *et al.*, 2009b). Those parasites did not cause mortality but could reduce fish flesh quality.

Aside from the parasite infections, bacterial pathogens *Edwardsiella ictaluri*, *Edwardsiella tarda, Flavobacterium columnare*, and *Aeromonas hydrophila* have been responsible for the significant mortality in striped catfish farms (Crumlish *et al.*, 2010, Panangala *et al.*, 2007, Shetty *et al.*, 2014). To the best of our knowledge, bacterial *E. tarda* has not been documented in striped catfish farms in Vietnam, although Shetty *et al.* (2014) reported that they appeared in striped catfish farms in India. Typically, *Edwardsiella ictaluri*, a causal agent of Bacillary Necrosis Pangasius (BNP), is currently the most economically serious pathogen in intensively reared striped catfish from the Mekong Delta (Crumlish *et al.*, 2002, Ferguson *et al.*, 2001). A survey of Phan *et al.* (2009) showed that BNP frequently occurred in most of the striped catfish farms (98% of farms) and caused up to 90% mortality. The *E. ictaluri* caused the mortality in all stages of striped catfish, although fingerlings and juveniles seem to be more sensitive than the adult stage (Dung *et al.*, 2008). In striped catfish infected with *E. ictaluri*, external clinical signs were found such as pale gills and ulcerative skin lesions (Ferguson *et al.*, 2001, Crumlish *et al.*, 2010, Dung *et al.*, 2012). The gill epithelium, gastrointestinal tract and skin are primary entry portals of *E. ictalurid* in striped catfish (Dung *et al.*, 2012, Pirarat *et al.*, 2016). Pirarat *et al.* (2016) also observed that *E. ictaluri* could persist up to 1 month in necrotic-participating phagocytic cells and in melano-macrophage centers. Similarly, *A. hydrophila* is also considered as major pathogen, causing huge economic losses in striped catfish farms (Subagja *et al.*, 1999). *A. hydrophila* is an aetiological agent of motile aeromonad septicemia (MAS), which mostly caused mortality in freshwater fish species (Newman, 1993). The outbreak of MAS is frequently associated with stress due to poor environment or immunocompromise in hosts. The clinical signs in infected striped catfish are usually septicemia, abdominal dropsy and blood-tinged peritoneal fluid (Inglis *et al.*, 1993). *A. hydrophila* is also seen as an opportunistic pathogen, which could be released during stressful conditions, including bacterial challenge with another pathogen (Nusbaum and Morrison, 2002). In addition, *F. columnare* is one of the oldest bacterial pathogens, which caused columnaris disease in freshwater fish including striped catfish (Hawke *et al.*, 1981, Declercq *et al.*, 2013). In the United States, *F. columnare* was considered as the second important bacterial pathogen threatening in the catfish culture industry after *E. ictaluri* (Shoemaker et al., 2007).

4. Disease management

4.1. The use of antibiotics in striped catfish culture

Disease problems due to bacterial infections constitute the largest threats of successful aquaculture. Early management strategies to control the spreading of pathogens in a sustainable intensively cultured system, therefore, are highly recommended. During the last decades, antibiotics have been widely used in Vietnamese aquaculture, both on a preventive and curative basis, as a traditional therapeutic. A large number of antimicrobials were applied for preventing

and treating the disease in three main stages of culture including hatcheries, nursing, and grow-out ponds (Phu *et al.*, 2016). Antimicrobials for treatment in striped catfish farms have been mainly derived from live microorganisms or chemically synthesized compounds. Moreover, up to 70% of farmers resorted to antimicrobial susceptibility testing of bacterial pathogens. Approximately 68% of farmers used prophylactic antimicrobials for treating the bacterial infections in fingerlings. Enrofloxacin, florfenicol, beta-lactams, and a combination of sulphonamides and trimethoprim have been commonly used in striped catfish farms. Most nursery and grow-out farmers used antimicrobials to treat the BNP and MAS diseases.

In addition, a survey of Rico *et al.* (2013) also showed that antibiotics were typically administered via medicated feed and then fed to the striped catfish. A period of antibiotics therapeutic lasted usually about 3 and 8 days. The farmers referred to apply antimicrobials to treat the outbreak of infectious diseases, only 5% of cases reported as prophylactic methods. However, a recent survey of Ström *et al.* (2019) showed that 72% of the famers used antibiotics to treat the diseases in around 3 days, and the treatment period could be extended depending on the farmers' economic, or whether the fish recovered or not. In the case of antibiotic treatment failure, most of the farmers changed to apply another type of antibiotic. Notably, the survey data also indicated that all farmers perceived the risks associated with antibiotics. Although antibiotics were widely used in striped catfish farms, a recent report suggested that 70% of *E. ictaluri* strains were resistant to commonly used antibiotics (trimethoprim, oxytetracycline, and streptomycin) and displayed a reduced sensitivity to flumequine, oxolinic acid and enrofloxacin (Dung *et al.*, 2008). Moreover, the excessive use of antibiotics in aquaculture has caused many problems including the occurrence of resistant microbial strains, environmental pollution and drug accumulation in fish flesh (Haniffa, 2011, Dawood *et al.*, 2018). Thus, alternative methods need to be developed to successfully reduce the use of antibiotics in aquaculture.

4.2. Application of immunostimulants in striped catfish culture

4.2.1. Vaccination

In aquaculture, strengthenning the immune response is the primary promising methods of controlling infectious diseases in fish (Raa, 1992). In this situation, fish vaccination has been developed as one of the most important and effective approaches to prevent and control infectious pathogens. Today, several commercial vaccines are available for channel catfish (*Ictalurus punctatus*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), Japanese amberjack (*Seriola quinqueradiata*), tilapia (*Oreochromis niloticus* L.) and Atlantic cod (*Gadus morhua*) (Sommerset *et al.*, 2005). Moreover, the development of vaccines from inactivated bacterial pathogens has been demonstrated to be efficacious in fish. Effect of inactivated *A. hydrophila*, a model of infection and vaccination, was also observed in striped catfish. Fish were intraperitoneally injected with heat-killed *A. hydrophila* (1×10^9 CFU/mL mixed with adjuvant), and a subclinical dose of live *A. hydrophila* (i.e. 2.7×10^5 CFU/mL). The results showed that live or dead bacteria could significantly stimulate the striped catfish immune parameters (i.e. specific antibody titer, plasma lysozyme, plasma peroxidase activity,

and head kidney phagocytosis) in treated groups compared to control group at day 7 and day 14 post-injection. Subclinical doses of live *A. hydrophila* enhanced the overall immune responses earlier than killed *A. hydrophila* (Sirimanapong *et al.*, 2014). Vaccination strategies are possibly effective and economical in protecting the health of fish and aquaculture animals from various infectious agents (Dadar *et al.*, 2017). For some diseases, however, the productive vaccines need to afford varying degree of success and cost effective (Midtlyng *et al.*, 1996).

4.2.2 Probiotics

Considering these issues, the development of new dietary supplementation strategies in which various health and growth promoting compounds as probiotics, prebiotics, and synbiotics have been evaluated. The benefits of such supplements include improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, as well as increased immune responses (Pandiyan *et al.*, 2013). In striped catfish, the effects of dietary supplementation with *B. amyloliquefaciens* 54A and *B. pumilus* 47B on the growth, immunity, disease resistance against *E. ictaluri*, and the stress tolerance against ammonia were demonstrated (Truong Thy *et al.*, 2017). In the latter study, fish were fed with a mixture of two *Bacillus* strains (*B. amyloliquefaciens* 54A and *B. pumilus* 47B) at concentrations of 1×10^8, 3×10^8, and 5×10^8 CFU/g for 90 days. The results indicated that average weight gain, as well as the immune parameters (i.e. phagocytic activity, respiratory bursts, and lysozyme activity), and resistance capacity to *E. ictaluri* infection were significantly enhanced in probiotics group (5×10^8 CFU/g) compared to control group. Stress response with ammonia showed significantly lower mortality rate (25%, 20% and 27%) in fish fed probiotics at all three levels of 1, 3 and 5×10^8 CFU/g than in fish fed control diet (75%). Similarly, Boonanuntanasarn *et al.* (2019) also evaluated the effects of probiotic *Saccharomyces cerevisiae*-based diets (0, 10^6, and 10^8 CFU/g) on striped catfish. The *S. cervisiae*- supplemented diets significantly improved growth rate and feed conversion ratio (FCR) over 120 days of culture period. Humoral immune parameters including total immunoglobulin, lysozyme, and alternative complement activities were significantly increased in probiotic-based diets compared to control. However, *S. cervisiae* did not affect hematological indices and blood chemistry values (glucose, cholesterol, triglycerides, protein, albumin, blood urea nitrogen, chloride, calcium, magnesium, iron, and phosphorus).

4.2.3. Prebiotics and microbial derivatives

Aside from probiotics, prebiotics have also been successfully applied in aquaculture. Prebiotics could function as immunostimulants in various fish species (Song *et al.*, 2014) since to their capacity in improving growth, gut health, and immune responses (Ali *et al.*, 2017, Gelibolu *et al.*, 2018). A publication of Akter *et al.* (2016) demonstrated that oral administration of diets supplemented with 0.6% mannan oligosaccharide (MOS) improved growth, feed utilization, and digestive enzyme activities in juvenile striped catfish after 12 weeks of feeding. The total lactic acid bacteria density in fish guts was significantly higher in MOS groups, although the density did not show significant difference counts between MOS groups than those in the control. Moreover, several hematological and immune parameters namely red blood cells,

white blood cells, lymphocytes, granulocytes, total Ig content and lysozyme activity were significantly increased in fish fed 0.4, 0.6, and 0.8% of MOS after 2-weeks challenged with *A. hydrophila.* The survival rate was significantly higher in 0.6 and 0.8% MOS groups compared to control diet after 3 weeks of bacterial injection (Akter *et al.*, 2019). Two prebiotics including oligochitosan and oligo-β-glucan were potential as immunostimulants and growth promoters in striped catfish. After 45 days of feeding trials, diets supplemented with single or a combination of oligochitosan or/and oligo-β-glucan significantly enhanced weight gain, specific growth rate, phagocytic and lysozyme activities as well as reduced mortality in striped catfish against *E. ictaluri* infection. Furthermore, the mixture of oligochitosan and oligo-β-glucan at 50 mg/kg feed- enriched diet was the best result of increasing weight gain (~26%) and reducing mortality (~38%) compared with the control group (Nguyen *et al.*, 2017). Another experiment of Hahor *et al.*, (2019) supplied various oligosaccharide types e.i. xylooligosaccharide (XOS), galactooligosaccharide (GOS), fructooligosaccharide (FOS) and MOS into fish diets in order to improve growth performance, gut health and immune response in hybrid catfish (*P. gigas* × *P. hypophthalmus*). These authors found that COS, FOS, and MOS at 0.6% significantly reduced fish conversion rate (FCR) after 10 weeks of feeding. Prebiotics, namely XOX, FOS, MOS, had significant effects on specific activities of digestive enzymes, but it depended on the type of stimulants. Significant increase in villus length was observed in the MOS group, while the villus length/crypt depth ratio was also increased in both XOS and MOS groups compared to control. Several hematological and immune parameters such as white blood cells, hemoglobin, lymphocytes, Ig, and lysozyme activity, increased in the MOS-based diet, while lesser improvements in at least one parameter were observed with FOS and XOS treatments. The mortality after *E. ictaluri* infection was significantly lower in the MOS-based diet as compared to other diets. A recent study of Tamamdusturi and Yuhana. (2016) also demonstrated the positive effect of microencapsulated probiotic *Bacillus* sp. NP5 (1%), prebiotic MOS (0.2%) and their combination (symbiotic)- based diets on growth performances, immune responses and resistance to *A. hydrophila* infection on striped catfish.

Moreover, β-glucans, is a heterogeneous group of glucose polymers containing β-(1,3)-linked β-d-glucopyranosyl units and β-(1,6)-linked side chains of varying lengths, which has been applied widely in striped catfish as immunostimulants. After 9 weeks of feeding β-glucans-based diets at different concentrations (0, 0.5%, 1% and 2%), the fish mortality was significantly improved after a cold challenge test at 15^{o}C in β-glucans treatments compared to control treatment (Soltanian *et al.*, 2014). A variety of immune parameters was examined in striped catfish after 0, 1, 3, 7, 14, 21 and 28 days feeding with fungal-derived β-glucans-based diets (0, 0.05, 0.1, or 0.2 %). The results showed that the lowest dose of fungal-derived β-glucan (0.05%) was insufficient to stimulate the immune response of the fish, while the high and medium doses enhanced respiratory burst activity, lysozyme activity, plasma anti-protease activity, natural antibody titres, and complement activity in time dependent manner in fungal-derived β-glucan groups compared to the control group (Sirimanapong *et al.*, 2015a). Moreover, some cytokine genes related to immune responses (transferrin, C-reactive protein, precerebellin-like protein, complement C3, and factor B, 2a MHC class II and interleukin-

1beta) in liver, kidney and spleen did not differ between dietary groups after 14 days of feeding. However, differential expressions of those examined genes were observed in infected fish compared to uninfected fish with *E. ictaluri* (Sirimanapong et al., 2015b).

Bich Hang *et al.* (2016) studied the effects of oral administration of *Escherichia coli* lipopolysaccharide (LPS) on the immune system of striped catfish. In this study, striped catfish were fed diets containing different LPS concentrations (0%, 0.01% and 0.05%) for 2 weeks and then the fish were continuously fed control diet for 4 weeks. Plasma cortisol and glucose were rather low and did not significantly differ among treatments. The lysozyme activity significantly increased in fish treated with LPS in weeks 2, 4 and 6. Fish fed 0.01% LPS displayed the highest values of respiratory burst activity at week 4. The total antibody significantly differed between 0.01% LPS- based diet and the control in week 2 and 4. The cumulative mortality was lower in LPS groups compared to the control group in the three different sampling times. In addition, intraperitoneal administration of LPS at 3, 15 or 45 mg LPS/kg fish also improved the striped catfish immune response. Indeed, the levels of plasma and spleen lysozyme, complement activity and antibody titers were significantly increased in fish injected with 3 mg LPS/kg fish. The striped catfish mortality was also significantly reduced in treatments treated with all doses of LPS. Differential over-expression of immune proteins such as complement component C3 and lysozyme C2 precursor were also induced in peripheral blood mononuclear cells of striped catfish (Bich Hang *et al.*, 2013). Moreover, these authors also compared the respective efficiency of LPS and levamisole with the one of antibiotic treatment- doxycycline after infection of fish by the bacteria *E. ictaluri*. Fish were injected with LPS (3 mg/kg fish), levamisole (5 mg/kg fish) or phosphate buffer saline as control. Non-specific immune variables (respiratory burst, lysozyme and complement activities) were significantly enhanced in stimulant groups compared with control at 21 days post injection. Respiratory burst and complement activities in levamisole groups were significantly higher than those in LPS groups. After *E. ictaluri* injection, accumulated mortality was significantly reduced in both non-antibiotic and antibiotic subgroups of LPS and levamisole compared with control. No differences in mortality were observed between fish treated with levamisole or LPS, and control fish treated with and without antibiotics (Bich Hang *et al.,* 2014).

4.2.4. Vitamins

Vitamin C (ascorbic acid) has an extensive role in the enhancement of growth and immunity in fish (Khan *et al.*, 2015, Asaikkutti *et al.*, 2016). In striped catfish, the ascorbic acid-based diets at different concentrations (i.e. 17.5, 35, 70, 175, 350 and 700 mg/kg feed) significantly stimulated growth, survival, body composition and metabolic enzymes activities after 60 days of feeding. The optimal dose was observed in fish fed 35 mg/kg ascorbic acid-based diet, which maximally enhanced weight gain, specific growth rate, protein efficiency ratio, and feed conversion (Daniel *et al.*, 2018). A study of Gopan *et al.* (2018) highlighted the effects of two concentrations (150 and 300 mg/kg) of carotenoids (i.e. astaxanthin, beta-carotene, canthaxanthin) on the nonspecific immune responses of *P. hypophthalmus* fingerlings. The results showed that total erythrocyte count, hemoglobin, respiratory burst, lysozyme activities were only enhanced in astaxanthin fed groups. Striped catfish injected with 1000 mg vitamin

C/kg fish significantly enhanced the respiratory burst activity, lysozyme, and total Ig, as well as reduced the mortality at day 17 and day 21 (Bich Hang, 2013).

5. The immune system in fish

The immune system of fish is very similar to that of other vertebrates; however, some immune molecules and organs vary between fish species and higher vertebrates such as mammals (Palanisamy *et al.*, 2018). The immune system of teleost fish originates from head kidney spleen, thymus, and scattered mucosal tissues of skin, gill, gonads, and gut (Smith *et al.*, 2019). Among the tissues, head kidney is the most vital immune organ vertebrates and exists as a central site of haematopoiesis until adult (Meseguer *et al.*, 1995, Zapata *et al.*, 2006). Similar to mammals, fish possess a set of immune cells including lymphocyte populations (T cells and B cells), neutrophils, monocytes/macrophages and plasma cells (Bayne and Gerwick, 2001).

In general, the fish immune system is divided into 2 subsystems- the innate immune system and the adaptive one. Fish strongly depend on innate system to inhibit or destroy the infection of pathogens, while their adaptive immune system is rather limited. The innate immune system can be categorized into three defence mechanisms: physical barriers, such as the skin and gastrointestinal tract, cellular components, such as pattern recognition receptors and immune cells including macrophages and neutrophils, and humoral components, such as the complement system, clotting system, anti-proteases, metal-binding proteins, lectins, lysozymes, antimicrobial peptides, opsonins. The specific components of the adaptive immune system include immunoglobulins, B cells, T cells, immunoglobulins, and major histocompatibility complex (Magnadóttir, 2006, Bayne and Gerwick, 2001).

Table 1. Cells and effector molecules of the adaptive and innate system of aquatic animal (Bayne and Gerwick, 2001).

Kind	Adaptive	Innate
Cells	T cells, B cells	NK cells, monocytes/macrophages, granulocytes (predominantly
Tissues	lymphoid	liver, spleen
Regulators	cytokines	cytokines
Humoral	immunoglobulins	Complement system, clotting system, anti-proteases, metal-binding
Kinetics	slow	fast

5.1. Physical barriers of the immune system

Physical barriers in fish include the skin (e.g., scales and mucus), gills, and epithelial layers of the gastrointestinal tract. Among them, the skin is extremely important in early prevention of pathogen invasion. In addition, skin mucus from teleost fish contains a combination of lectins, lysozymes, complement proteins, and antimicrobial peptides (AMPs); all of which play a critical role in neutralizing pathogens (Esteban, 2012, Fast *et al.*, 2002).

5.2. Cells involved in immune system

Cells involved in the immune system of fish include T cells, B cells, macrophages and other leucocytes. T cells are activated by the interaction with antigens that lead to their differentiation into T-helper cells and T cytotoxic cells. Activated T helper cells in turn activate the B cells and macrophages, thus leading to the initiation of adaptive immune machinery (Abbas *et al.*, 2007). Similarly, B cells present in the spleen and kidney are responsible for the production of immunoglobulins, including IgM, IgD, IgT and IgZ (Wilson *et al.*, 1997, Stenvik and Jørgensen, 2000, Danilova *et al.*, 2005, Hansen *et al.*, 2005). Macrophages and polymorphonuclear leucocytes such as neutrophils are involved in phagocytosis. They produce enzymes such as lysozyme, peroxides and other free radicals which destroy the invading pathogens (Secombes and Fletcher, 1992, Fischer *et al.*, 2003).

5.3. Phagocytosis

One typical innate immune defence mechanism is phagocytosis by phagocyte cells which are principally dedicated to the recognition and elimination of invading organisms (Dussauze *et al.*, 2015). Phagocyte cells include monocytes, macrophages, neutrophils, mast cells (MCs), dendritic cells (DCs) and non-specific cytotoxic cells (Neumann *et al.*, 2001, Esteban *et al.*, 2015). Recent accumulating data show that phagocyte cell activity in fish could be induced by immunostimulants, a natural or chemical substance that stimulates the immune system (Vallejos-Vidal *et al.*, 2016) in fish principally meant to enhance the innate immune response mechanisms (Sakai, 1999) as well as inducing pronounced physiological effects on growth. Moreover, in the innate immune system, macrophages of several teleost fish species have been demonstrated to destroy pathogens through phagocytosis, the production of reactive oxygen species (ROS) and nitric oxide (NO), and the release of several inflammatory cytokines and chemokines, similar to mammalian macrophages (Grayfer *et al.*, 2018, Nathan and Xie, 1994). Roca et al (2013) and Ru et al. (2015) demonstrated that the increased generation of ROS appeared to be accompanied with the bacterial-killing activity (Roca and Ramakrishnan, 2013, Lu *et al.*, 2015). ROS commonly measured by respiratory burst assay (RBA). Previous studies indicated that the RBA and NO could act as indicators of innate immune responses (Gobi *et al.*, 2016), Omitoyin *et al.*, 2019), Yin *et al.*, 2006), Fawole *et al.*, 2016).

5.4. Innate immune responses

5.4.1. Humoral innate immune responses

Lysozyme

Lysozyme is an important defence molecule of the innate immune system, which is important in mediating protection against microbial invasion. It splits the β (1→4) linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell walls (peptidoglycan layers) of Gram-positive bacteria, thus preventing them from invading. Gram-negative bacteria are not directly damaged by lysozyme. However, when the outer cell wall of Gram-negative bacteria is disrupted due to the action of complement and other enzymes exposing the inner peptidoglycan layer of bacteria, then lysozyme becomes effective. Lysozyme is present in mucus, lymphoid tissue, and serum of most fish species (Nigam *et al.*, 2012, Bergsson *et al.*, 2005, Magnadóttir *et al.*, 2005). Besides an antibacterial function, lysozyme also promotes phagocytosis by

directly activating polymorphonuclear leucocytes and macrophages or indirectly by an opsoninic effect. High activities of the enzymes are found against *Micrococcus lysodeikticus* in the lymphomyeloid tissues of elasmobranchs and in the plasma of teleost. Like mammals, the lysozyme in fish occurs mainly in neutrophils, monocytes and a small amount in macrophages (Saurabh and Sahoo, 2008). There are two types of lysozyme including c- and g-type that have been reported in vertebrates, which have divergent amino acid compositions, molecular weights and enzymatic properties (Prager, 1996, Irwin and Gong, 2003). Recombinant c-type and g-type lysozymes of Japanese flounder have been shown to have high bacteriolytic activity against *Pfiesteria piscicida* and *V. anguillarum* but low activity against *E. tarda* and β-haemolytic *Streptococcus* sp., which are pathogenic agents of the Japanese flounder (Minagawa *et al.*, 2001). In the Atlantic cod, an absence of c-type lysozyme genes was demonstrated; however, four g-type lysozyme genes were identified in several different tissues and compensated for the lack of c-type lysozymes (Seppola *et al.*, 2016). Moreover, the levels of lysozyme in skin mucus differentially vary depending on the fish species and environmental conditions (Nigam *et al.*, 2012). In addition, the levels of lysozyme in skin mucus of seawater fish species are higher than those of fish inhabiting fresh waters (Subramanian *et al.*, 2007).

Complement system

Most of the organisms respond to the pathogens by activating their immune system, which is performed by specific molecules termed as complements. As in higher vertebrates, fish possess three different pathways of complement system, namely activated by antibody-antigen complexes and thus a bridge between innate and adaptive immunity; the alternative pathway, which is independent of antibodies and activated directly by pathogens; and the lectin pathway which is activated by the binding of the mannose-binding lectin, or ficolin, to mannose residues present on the pathogen surface (Nonaka and Smith, 2000, Zarkadis *et al.*, 2001, Boshra *et al.*, 2006). All pathways could activate the C3 convertase, which cleaves inactive C3 into C3a, that acts as a chemotactic factor and aids in inflammation, and C3b, which acts as an opsonin (Dunkelberger and Song, 2010, Chondrou *et al.*, 2006). These components are involved in phagocytosis, respiratory burst and inflammation (Sunyer *et al.*, 2003). Apart from being involved in the innate immune response, C3b component also plays a major role in adaptive immune system as well. The C3b component adheres to the surface of the pathogen and further links with the complement receptors available in antigen-presenting cells including B cells. These cells process the antigen and provide it to T cells via major histocompatibility complex that leading to adaptive immune response (Fearon *et al.*, 2000).

Antimicrobial peptides

Antimicrobial peptides, also known as host defence peptides, are generally oligopeptides with a varying number of amino acids that are generally positively charged and play a major role in the innate immune system. Antimicrobial peptides protect against a variety of pathogens via disruptive or pore-forming actions against bacterial membranes. Over 90 fish AMPs have been identified and are characterized as β-defensins, cathelicidins, hepcidins, histone-derived

peptides and fish-specific piscidins (Lazarovici *et al.*, 1986, Lemaître *et al.*, 1996, Cuesta *et al.*, 2011, Browne *et al.*, 2011). Of the antimicrobial peptides, β-defensin was identified in gilthead seabream and possessed antimicrobial activity against *V. anguillarum*. Similarly, β-defensin has shown an inhibitory effect on the growth of *Escherichia coli* DH5α and *Streptococcus agalactiae* in Nile tilapia (Dong *et al.*, 2015).

5.4.2. Cytokines involved in innate immune responses

Initiation of the innate immune response begins when germline-encoded intracellular or extracellular pattern recognition receptors (PRRs) of an immune cell bind to a pathogen associated molecular patterns PAMP found on a pathogen. Toll-like receptors were the first PRRs to be discovered in fish. TLRs identified in fish including TLR2, TLR5M, TLR5S, TLR9, and TLR21, which could specifically recognize PAMPs from bacteria, while TLR1, TLR4, TLR14, TLR18, and TLR25 may also be sensors of bacteria (Zhang *et al.*, 2014). Several studies in fish have provided direct evidence suggesting that tumor necrosis factor (TNF) TNF-α and -β are important in activating of macrophages, leading to increased respiratory activity, phagocytosis and nitric oxide production (Yin *et al.*, 1997, Mulero and Meseguer, 1998, Tafalla *et al.*, 2001). In *Mycobacterium marinum*-infected zebrafish, TNF-α was shown to promote macrophage survival and also restrict bacterial growth in infected macrophages (Clay *et al.*, 2008). Roca *et al.* (2008) also suggested that TNFα was mainly involved in the recruitment of leukocytes to the inflammatory. Fish TNF-α exerted pro-apoptotic activity, which could trigger apoptosis in dose and target cell dependent. In tilapia, TNF-α has been shown to upregulate granzyme expression in non-specific cytotoxic cells and provides protection of these cells from activation-induced cell death triggered apoptosis is likely to be dose and target cell dependent.

Similarly, the interleukins are also responsible for bathing the infectious areas with granulocytes and macrophages while further secreting additional cytokines to cleanse the region. Among the interleukins, IL-1β is one of the earliest expressed pro-inflammatory cytokines and enables organisms to respond promptly to infection by inducing a cascade of reactions leading to inflammation (Reyes-Cerpa *et al.*, 2012). IL-1β is produced by a wide range of cell types after activation of host PRRs by PAMPs or danger associated molecular patterns (DAMPs) (Angosto *et al.*, 2012, Chaves-Pozo *et al.*, 2004, Pelegrın *et al.*, 2004). IL-1β also induced severe gut inflammation and expression of *TNFα* (Bo *et al.*, 2015). Several fish IL-1βs also modulate expression of IL17 family members, important for antibacterial defence (Secombes *et al.*, 2011, Kono *et al.*, 2011, Wang *et al.*, 2014). IL-1β has also been shown to enhance antibody production when administered with bacterial vaccines, suggesting it may be exploited as an immune-adjuvant for improving vaccine efficacy (Taechavasonyoo *et al.*, 2013, Yin and Kwang, 2000). IL-1β induced the expression of *IL6* and *COX2* can be inhibited by the stress hormone cortisol (Castro *et al.*, 2011). Teleost IL-1β has been found to be regulated in response to various stimulants (Lee *et al.*, 2006, Corripio-Miyar *et al.*, 2007). In addition, it has been demonstrated that IL-6 is also involved in the cascade leading to an inflammatory response (Savan and Sakai, 2006). Interferon genes are involved in mediating cellular resistance against viral pathogens and modulating innate and adaptive immune

systems. IFNs are classified into two main groups called type I and type II (Savan and Sakai, 2006). Type I IFN includes the classical IFNα/β, which is induced by viruses in most cells, whereas type II IFN is only composed of a single gene called IFNγ and is produced by natural killer cells and T lymphocytes in response to IL-12, IL-18, mitogens or antigens (Samuel, 2001).

5.5. Adaptive immune responses

Similar the innate immune system, the adaptive immune system includes both humoral and cellular components. B cells are key elements of the humoral adaptive immune response and produce high affinity immunoglobulin against foreign antigen. Moreover, T cells are key elements of cellular adaptive immunity, which can be activated to function as a helper (CD4+) T cell, a regulatory (CD4+) T cell or a cytotoxic (CD8+) T cell (Smith *et al.*, 2019). Major histocompatibility complex (MHC) molecule presents these lysed peptides to the T cell, in order to bring in the adaptive immunity to specific pathogen. MHC I initiates cellular immunity through activating CD8+ receptor which has the cytotoxic T cell, whereas MHC II activates the humoral immunity through CD4+ Thelper cell. Most of the host cells express MHC I receptor, whereas MHC II receptors are expressed only in the professional antigen-presenting cells (Palanisamy *et al.*, 2018). Earlier it was believed that only one type of antibody such as IgM was present in fish; many studies have shown the existence of other antibodies, such as IgD, IgZ and IgT. Although the predominant Ig molecule in teleost is IgM, the second identified antibody is IgD (Palanisamy *et al.*, 2018).

6. Application of plant extracts as immunostimulants in aquaculture

The immunostimulants mentioned above have successfully enhanced immune responses and defense mechanisms in striped catfish, acting as diet additives and thus preventing losses from diseases. Moreover, synthetic or natural immunostimulants (i.e. probiotics, complex carbohydrates, nutritional factors, hormones, cytokines, products derived from animal, plants and algae) have successfully promoted fish growth, innate immune response (lysozyme, complement, phagocyte activity, respiratory burst and microbial activities of phagocytes), as well as adaptive immune response (immunoglobulin production) (Anderson, 1992, Galeotti, 1998, Sakai, 1999). However, these immunostimulants may produce side effects in fish and can be potentially dangerous for consumers due to the residues (Bulfon *et al.*, 2015). Therefore, a growing interest has emerged for plant-derived products as a promising approach complementary to vaccination and traditional drugs, as they are easy to access and cheap sources, effective in treatment (Mohamad and Abasali, 2010). Boosting the immune response with ecological compounds is an effective strategy to promote sustainable aquaculture. From this perspective, bio-products resulting from natural plants have attracted considerable attention as a source of eco-friendly prophylactic compounds for using in the aquaculture industry, which may contribute to sustainable aquaculture.

6.1. Sources of plant extracts

Medicinal plants have been reported to possess a broad spectrum of growth promotion, appetite stimulation, antimicrobial, immunostimulant, anti-inflammatory, anti-stress, anti-cancer

properties in human and they have been known around the world for thousands of years as traditional medicines. In several Asian countries such as China, India, Japan, Thailand, Korea, Vietnam and some countries in South and Central America, medicinal plants have been used to prevent and treat bacterial, fungal and viral diseases in human and veterinary medicine (Briskin, 2000, Lovkova *et al.*, 2001, Banskota *et al.*, 2003, Sher, 2009, Wallace *et al.*, 2010, Hashemi and Davoodi, 2011, Sakarkar and Deshmukh, 2011, Sharma *et al.*, 2011, Bueno Pérez *et al.*, 2014). Vietnam has at its disposal about 3780 species that possess medicinal properties (Trang Thi Huong Tran and Dang Thu Nguyen, 2016), and potentially provide plenty of botanical sources for aquaculture treatment.

In aquaculture, the application of medicinal herbs was reported in various Asian countries (Direkbusarakom, 2004). Recently, more than 60 different plant species have been assessed for their capacities on anti-stress, growth promotion, anti-pathogen, improving immune response in aquaculture, which were widely investigated in folk medicine in China, India, Thailand, and Korea (Bulfon *et al.*, 2015). The medicinal plants have been generally administered as powders of plant parts (seeds, bulbs, leaves) or plant-derived products, including crude extracts by aqueous or organic solvents (ethanol, methanol, ethyl acetate, hexane, butane, acetone, benzene, petroleum ether, etc.), or other preparations such as essential oils, concoctions and decoctions.

6.2. Extract solvents and bioactive constituents in aquaculture

The solvents used for the extraction (water, methanol, ethanol, ethyl acetate, hexane, butane, acetone, benzene, petroleum ether, etc.) is one of the factors that influence to the yield of constituents, and resulting in the differential effects of antibacterial and immunomodulatory properties in fish. Divyagnaneswari *et al.* (2007) demonstrated that the hexane soluble fraction of *S. trilobatum* was more protective than the water soluble fraction when administered intraperitoneally to Nile tilapia. Similarly, the ethanol extract of guava appeared more effective as an immunostimulant in Nile tilapia than aqueous extract (Gobi *et al.*, 2016). Moreover, an aqueous extract inhibited the Toll-like receptors (TLRs)-mediated signaling pathways, while an ethanolic extract from the same plant activated the TLR signals. Similarly, the injection of triherbal aqueous, ethanol or methanol solvent leaf extracts from *A. indica*, *O. sanctum* and *C. longa* enhanced non-specific immune parameters and disease resistance against *A. hydrophila* in goldfish, but the ethanol solvent extract appeared more effective (Harikrishnan *et al.*, 2009b). Moreover, the functions of herbs vary depending on the species of herb, which may be due to the variation in herbal components. Some herbs induce anti-inflammatory responses (Vallejos-Vidal *et al.*, 2016), while others activate pro-inflammatory responses (Awad and Awaad, 2017). The extraction efficiency is partly affected by the type of solvents with varying polarity in the same conditions of pH, temperature as well as extraction time. Total phenol and flavonoid contents of rice paddy-*Limnophila aromatica* (Lamk.) Merr in pure ethanol extract were higher than that in pure acetone, pure methanol and water extracts, although the extraction yield was the highest in methanol and decreased in water, ethanol and acetone (Stalikas, 2007, Do *et al.*, 2014). The biological compounds in the extracts including phenols, gallic acid, myricetin were also variable after extraction in different solvents (Kumar *et al.*, 2013).

Moreover, methanol and ethanol were the best solvents for extraction of biological components, which mainly function in immunostimulatory properties and antibacterial activity (Guo *et al.*, 2001, Ashraf *et al.*, 2016).

Moreover, based on their chemical structure, plant active compounds can principally be categorized into alkaloids, terpenoids (triterpenes and steroid saponins), phenolic compounds, glycosides, flavonoids, tannins and polysaccharides (Lovkova *et al.*, 2001). Phenolic compounds contain flavonoids, tannins, and other phenols (Okwu and Okwu, 2004), which located in the cell vacuole and potentially toxic to the growth and development of pathogens, as well as enhances lymphocyte proliferation, B-cell proliferation, secretion of interferon, and phagocytic activity (Chiang *et al.*, 2003, Yadav *et al.*, 2005). Alkaloids are one of the most efficient and therapeutically significant plant substances (Okwu, 2005), which include nicotine, cocaine, morphine and codeine, quinine, reserpine (Okwu and Okwu, 2004). Alkaloids possess anti-tumor activity (vinblastine and vincristine), antimicrobial (cepharanthine), analgesic activity (morphine) and are also known to enhance immune response and a large number of alkaloids are being investigated for their immunostimulating properties (Brindha, 2016). Pharmaceutically important glycosides include saponins and anthracene derivatives, which mainly participate in the stimulation of the immune system and antimicrobial activity (Akbay *et al.*, 2003, Chiang *et al.*, 2003, Pandey *et al.*, 2005). Quercetin and rutin are among the most largely found flavonoids, which function as cancer prevention, anti-inflammatory and antiviral activities (Trugo *et al.*, 2003). Tannins are water-soluble high molecular weight polyphenolic compounds. Tannins possess many physiological activities such as stimulation of phagocytic cells, host-mediated tumor activity and a wide range of anti-infective action (Okwu and Okwu, 2004). Triterpenoids function in mediating immunological processes by enhancing antibody production and suppress T-cell response (Podder *et al.*, 2015).

In aquaculture, most of the medicinal plants have demonstrated their apparent ameliorating effects on immune responses and disease resistance. Interestingly, several studies were conducted to illustrate the potential effects of active compounds derived plants, as immunomodulatory and protective agents against infectious diseases in fish were described in table 1. Heartbreak grass (*Gelsemium elegans*)-derived alkaloids significantly improved the intestinal morphology, antioxidant, and upregulated the cytokines including *il1β*, *il8*, *tnfα* and *ifnα*, *trl1*, *3*, *4* and *7* in intestine of Wuchang bream (*Megalobrama amblycephala*) after 12 weeks of feeding (Ye *et al.*, 2019a, Ye *et al.*, 2019b). Alkaloids derived sanguinarine downregulated the expression of *il1β*, *il8*, and *tnfα*, whereas the sanguinarine could stimulate the lysozyme, complement C3 and glutathione peroxidase activities in Koi carp (*Cyprinus carpio*) 8 weeks post feeding (Zhang *et al.*, 2019b). Moreover, sanguinarine also acts as an adjuvant vaccine, which could be combined with inactivated ISKNV (Infectious spleen and kidney necrosis virus) vaccine to enhance the expression of *TNFα*, *IgM* and *Viperin*, inhibited the ISKNV replication, and significantly improve the survival rate in Chinese perch (*Siniperca chuatsi*) after challenge with ISKNV (Zhang *et al.*, 2019a). Saponins derived triterpenoid saponin, which were collected from Soap tree (*Quillaja Saponaria*) significantly improved the body weight in common carp and Nile tilapia at 8 weeks of feeding (Francis *et al.*, 2005);

improved the immune parameters (haemocyte count, respiratory burst (RBA), superoxide dismutase (SOD), and glutathione peroxidase (GPx), phagocytic activities), and survival rate in white shrimp (*Litopenaeus vannamei*) injected with *Vibrio alginolyticus* (Su and Chen, 2008); and increased the leukocyte phagocytic, alternative complement haemolysis (ACH50) in Turbot (*Scophthalmus maximus*) (Han *et al.*, 2014).

Heliotropium filifolium (Miers) Reiche derived terpenoids including filifolinone increased cytokines related to pro-inflammatory (*il1β*, *il8*, *tnfα*), antiviral (*ifnα*), and adaptive immunity (*il12*, *tgfβ1*) in Atlantic salmon (*Salmo salar*) in both *in vitro* and *in vivo* (Valenzuela *et al.*, 2013). Filifolinone also upregulated the expression of *ifn1*, *ifnγ*, *il4*/*13a* and *il17d*; whereas filifolinoic acid reduced T cell number and downregulated the mRNA of *ifnγ*, *il12*, *il4*/*13a* in rainbow trout (*Oncorhynchus mykiss*) kidney (Valenzuela *et al.*, 2016).

Administration of flavonoids and their derives also improved the growth performances, antioxidant (CAT and SOD), immune parameters (lysozyme, ACH50, total protein, bactericidal activity, IgM) and cytokines related to immune responses (*myd88*, *il1β*, *tnfα*, *mx1,* and *il8*) and improved the survival rate after challenge test in several fish species (Arciuli *et al.*, 2017, Awad *et al.*, 2015, Chakraborty *et al.*, 2015, Chi *et al.*, 2016, Kim *et al.*, 2013, Li *et al.*, 2019, Li *et al.*, 2016, Thawonsuwan *et al.*, 2010, Zhou *et al.*, 2015).

Chinese gallnut grass derived tannins based diets significantly enhanced final body weight and specific growth rate in European seabass after 8 weeks of feeding (Omnes *et al.*, 2017).

Pigments derived anthracenedione enhanced weight gain, feed intake, SGR, FCR, and blood indices (red blood cells, white blood cells, lymphocytes, monocytes, neutrophils, eosinophils), immune makers (Phagocytic activity, serum IgM, respiratory burst activity, lymphocyte proliferation, MPO, ACH50, lysozyme activity, nitric oxygen) in striped dwarf catfish (*Mystus vittatus*) after 30 days of oral administration (Harikrishnan *et al.*, 2019). In addition, carotenoids derived β-carotene also enhanced the immune parameters in common carp (*Cyprinus carpio*) and rainbow trout (Amar *et al.*, 2000, Amar *et al.*, 2004, Anbazahan *et al.*, 2014).

Table 2. Compounds classification and their immunopotential properties in aquaculture

Class	Subclass	Compounds	Plant	Species	Mechanism of action	Best dose	Administration /Duration	References
Alkaloids			Heartbreak grass (*Gelsemium elegans*)	Wuchang bream (*Megalobrama amblycephala*)	- Increase intestinal morphology (villus length, muscle thickness, and villus number) - Improved intestinal antioxidant (superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity and malondialdehyde levels) - Increase and *ifnα*, *trl1*, *3*, *4* and *7* in intestine, decrease *il10* and *tgfβ*	- 40 mg/kg - 20 and 40 mg/kg - 20 and 40 mg/kg	Oral/12 weeks	(Ye *et al.*, 2019b) (Ye *et al.*, 2019a)
		Sanguinarine	Bloodroot (*Sanguinaria canadensis*)	Koi carp (*cryprinus carpiod*)	- Decrease *il1β* in serum - Decrease *il1β* and *tnfα* in intestine - Decrease *il8* in serum - Increased lysozyme, complement C3 and glutathione peroxidase - Increase SOD - Decrease malondialdehyde	- 50 mg/kg - 50, 150, or 450 mg/kg - 50 and 150 mg/kg - 50, 150, or 450 mg/kg - 50 mg/kg - 50 and 150 mg/kg	Oral/8 weeks	(Zhang *et al.*, 2019b)
		Anisodamine + inactivated ISKNV vaccine (Infectious spleen and kidney necrosis virus)	Solanaceae plant	Chinese perch (*Siniperca chuatsi*)	- Increase *tnfα* and *Viperin* in spleen and mesonepgron - Increase *IgM* in mesonepgron. - Inhibited the ISKNV replication in spleen and mesonepgron. - Improve the survival rate post challenge with ISKNV	- 200 µg/fish	Injection/ 1, 4, 7, 10, 14, 15, 20, 23 days	(Zhang *et al.*, 2019a)

Table 2. Compounds classification and their immunopotential properties in aquaculture

Class	Subclass	Compounds	Plant	Species	Mechanism of action	Best dose	Administration /Duration	References
Saponins	Triterpenoid saponin		Soap tree (*Quillaja Saponaria*)	White shrimp (*Litopenaeus vannamei*)	- Increase total immunoglobulin, - Phenoloxidase activity - Increase respiratory burst (RBA), SOD, and glutathione peroxidase (GPx) activity - Increase phagocytic activity - Improve survival rate after challenge with *Vibrio alginolyticus*	- 0.5, 1 and 2 mg/L - 2 mg/L - 1 and 2 mg/L - 0.5, 1 and 2 mg/L - 0.5, 1 and 2 mg/L	Immersion/ 24, 48 and 72h	(Su and Chen, 2008)
			Soap tree (*Quillaja Saponaria*)	- Common carp (*Cyprinus carpio* L.) - Nile tilapia (*Oreochromis niloticus* L.)	- Improve body weight	- 150 mg/kg - 300 mg/kg	Oral/8 weeks	(Francis *et al.*, 2005)
			Soap tree (*Quillaja Saponaria*)	Turbot (*Scophthalmus maximus*)	- Leukocyte phagocytic activity, ACH50, - Improve antibacterial activity	- 15 and 35 mg /L	Immersion/ 6, 12, 24, 48 and 72h	(Han *et al.*, 2014)
	Triterpenoid saponin + inactivate vaccine (*Vibrio anguillarum*)		Soap tree (*Quillaja Saponaria*)	Turbot (*Scophthalmus maximus*)	- Improve survival rate after challenge with *Vibrio anguillarum* - Increase *IgM*	- 45 mg/L	Immersion/7, 14 and 28 days	(Wang *et al.*, 2016)
Terpenoids		Filifolinone and Filifolinyl senecionate	*Heliotropium filifolium* (Miers) Reiche	Chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line	- Inhibited the infectious pancreatic necrosis virus (IPNV)	- 10-120 µg/mL	Cell stimulation/ 3 days	(Modak *et al.*, 2010)

Table 2. Compounds classification and their immunopotential properties in aquaculture

Class	Subclass	Compounds	Plant	Species	Mechanism of action	Best dose	Administration /Duration	References
		Filifolinone	*Heliotropium filifolium* (Miers) Reiche	- Monolayers of SHK-1 cells Atlantic salmon (*Salmo* salar)	- Increase cytokines related to pro-inflammatory (*il1β*, *il8*, *tnfα*), antiviral (*ifnα*), and adaptive immunity (*il12*, *tgfβ1*) in cells	- 5 µg/mL	Cell stimulation/ 24h	(Valenzuela *et al.*, 2013)
				- Atlantic salmon	- Increase cytokines related to pro-inflammatory (*il8*, *tnfα*), antiviral (*ifnα*), and adaptive immunity (*ifnγ*, *il12*, *tgfβ1* and *il10*) in kidney and no significant change in spleen	- 100 µg/fish	Injection/48h	
		Filifolinone and Filifolinoic acid	*Heliotropium filifolium* (Miers) Reiche	Rainbow trout (*Oncorhynchus mykiss*)	- Upregulation of *ifn1*, *ifnγ*, *il4/13a* and *il17d* in kidney - Downregulation of *ifnγ*, *il12*, *il4/13a*; reduce T cells	- 100 µg/fish	Injection/48h	(Valenzuela *et al.*, 2016)
Flavonoids			*Allium mongolicum* Regel	Snakehead (*Channa argus*)	- Enhance growth, antioxidant. - Increase serum AKP, lysozyme activity, C3, IgM concentration, liver antioxidant enzymes activity (SOD, CAT and GSH-Px) - Decreased serum cortisol, liver MDA content, serum ALT and AST activity - Upregulation of cytokines (*sod*, *igm*, *hsp70*, *hsp90*, *iκbα* and *gr* in head kidney - Downregulation of *nfκb* p65 and inflammatory cytokines (*tnfα*, *il1β* and *il8*) in head kidney	- 10, 20 or 40 mg/kg	Oral/ 28 and 56 days	(Li *et al.*, 2019)

Table 2. Compounds classification and their immunopotential properties in aquaculture

Class	Subclass	Compounds	Plant	Species	Mechanism of action	Best dose	Administration /Duration	References
					- Increase the survival rate after challenge with *Aeromonas hydrophila*			
	Isoflavones		Soya bean *(Glycine max)*	Golden pompano (*Trachinotus ovatus*)	- Increase weight gain (WG) and specific growth rate (SGR)	- 40 mg/kg	Oral/8 weeks	(Zhou *et al.*, 2015)
					- Increase plasma total protein content, complement 3 content, lysozyme activity as well as respiratory burst activity	- 40 mg/kg		
					- Increased plasma alkaline phosphatase activity, hepatic total antioxidative capacity, CAT and SOD activity	- 40 or 60 mg/kg		
					- Increase *hsp70* expression	- 40, 60 and 80 mg/kg		
					- Improve survival rate after challenge with *Vibrio harveyi*	- 20, 40, 60, and 80 mg/kg		
		Genistein		- Nile tilapia, (*Oreochromis niloticus*)	- Increase phagocytic and respiratory burst activities	- 1 g/kg	Oral/35 days	(Chakrabort y *et al.*, 2015)
		Daidzein	Soya bean *(Glycine max)*	- Gibel carp (*Carassius auratus gibelio*)	- Increase respiratory burst - Increase lysozyme activity - CAT and SOD	- 200 and 400 mg/kg - 40 mg/kg - 40, 200 and 400 mg/kg	Oral/80 days	(Li *et al.*, 2016)
	Flavonols	Dihydroquercetin	Deodar (*Cedrus deodara* Roxb.)	Gilthead seabream (*Sparus aurata* L.)	- Increase phagocytosis, respiratory burst, ACH50, total protein, peroxidase, bactericidal activity and IgM level	- 0.1%	Oral/14 days	(Awad *et al.*, 2015)

Table 2. Compounds classification and their immunopotential properties in aquaculture

Class	Subclass	Compounds	Plant	Species	Mechanism of action	Best dose	Administration /Duration	References
		Quercetin + Spirulina		Olive flounder (*Paralichthys olivaceus*)	- Increase lysozyme activity	- 6.8%	Oral/10 weeks	(Kim *et al.*, 2013)
		Quercetin	Nettle (*Urtica dioica*)	Rainbow trout (*Oncorhynchus mykiss*)	- Increase lysozyme, total protein, antiprotease and bactericidal activity - Increase total serum IgM	- 1% - 0.1 and 0.5%	Oral/14 days	(Awad *et al.*, 2013)
		Kaempferol	Wood fern (*Dryopteris crassirhizoma*)	- Grass Carp (*Ctenopharyngodon Idella*) head kidney macrophages	- Upregulation of cytokines (*myd88*, *il1β*, *tnfα*, and *Mx1*)	- 1 and 10 μg	- Cell stimulation 2, 8, 12, and 24 h	(Chi *et al.*, 2016)
				- Grass Carp	- Increase lysozyme activity, complement C3, SOD, phagocytic activity, and IgM level - Improve survival rate after challenge with *A. hydrophila*	- 0.1, 1, and 5 μg/fish - 1 μg/fish	- Injection/1, 2, and 3 weeks	
	Flavanols	Epigallocatechin gallate	Green tea (*Camellia sinensis*)	Rainbow trout (*Oncorhynchus mykiss*)	- Increase serum ACH50 and lysozyme activities	- 20 and 100 mg/kg	Oral/8 weeks	(Thawonsuwan *et al.*, 2010)
		Catechin and epigallocatechin	Canosina Nero di Troia (*Vitis vinifera*)	Sea bass (*Dicentrarchus labrax* L.)	- Tyrosinase and peroxidase activity	- 100 and 200 mg/kg	Oral/8 months	(Arciuli *et al.*, 2017)
Tannins		Tannic acid	Chinese gallnut grass	European seabass (*Dicentrarchus labrax*)	- Increase final body weight and Specific growth rate	- 20 and 30 g/kg	Oral/8 weeks	(Omnes *et al.*, 2017)
Pigments		Anthracenedione	Golden shower (*cassia fistula*)	Striped dwarf catfish (*Mystus vittatus*)	- Improve weight gain, feed intake, SGR, FCR - Increase red blood cells, white blood cells, lymphocytes	- 2mg/kg - 1, 5 and 10 mg/kg	Oral/30 days	(Harikrishnan *et al.*, 2019)

Table 2. Compounds classification and their immunopotential properties in aquaculture

Class	Subclass	Compounds	Plant	Species	Mechanism of action	Best dose	Administration /Duration	References
					- Increase monocytes, neutrophils, total protein	- 5 and 10 mg/kg		
					- Increase eosinophils, albumin, and globulin	- 5 mg/kg		
					- Phagocytic activity, serum IgM, respiratory burst activity, lymphocyte proliferation, MPO, ACH50, lysozyme activity	- 5 and 10 mg/kg		
					- SOD, nitric oxygen species (NOS)	- 5 mg/kg		
					- Reduce mortality against *A. hydrophila*	- 1, 5 and 10 mg/kg		
	Carotenoids	β-carotene		Common carp (*Cyprinus carpio*)	- Increase phagocytic activity and ACH50, reactive oxygen species (ROS), NOS, lysozyme activity	- 50 and 100 mg/kg	Oral/1, 2, and 4 weeks	(Anbazahan *et al.*, 2014)
					- Reduce mortality against *A. hydrophila*			
			Marine alga (*Dunaliella salina*)	Rainbow trout (*Oncorhynchus mykiss* Walbaum)	- Increase serum ACH50, lysozyme activity,	- 100 and 200 mg/kg	- Oral/9 weeks	(Amar *et al.*, 2004)
					- Increase SOD, phagocytic index, phagocytic rate, in kidney	- 200 mg/kg		
					- Increase total immunoglobulin, serum ACH50	- 200 and 400 mg/kg	- Oral/12 weeks	(Amar *et al.*, 2000)

5.3. Mode of actions of plant extracts in aquaculture

6.3.1. Plant extracts act as appetite stimulators and growth promotors

Many studies have demonstrated that plants and their derivatives could function as appetite stimulators and growth promotors (Reverter *et al.*, 2014, Van Hai, 2015). Hoseinifar *et al.* (2019a) observed that jujube (*Ziziphus jujuba* Mill.) fruit hydro-alcoholic extract (0.25, 0.5 and 1%) was effective as a growth promoter and appetite stimulator for common carp (*Cyprinus carpio*) where a significant increase in weight gain (WG), specific growth rate (SGR) and a significant decrease in FCR were observed after 8 weeks of feeding. Similarly, 2% coriander seed (*Coriandrum sativum*) methanol extract-based diets significantly enhanced the SGR, final weight (FW), and condition factor (CF) in rainbow trout (*Oncorhynchus mykiss*) compared with the control group after 8 weeks (Naderi Farsani *et al.*, 2019). A study of Van Doan *et al.* (2019) revealed that dietary supplemented with 0.2% Assam tea (*Camellia sinensis*) ethanol extract significantly enhanced final body weight, weight gain, and specific growth rate; while a decreased feed conversion ratio in Nile tilapia after 4 and 8 weeks of feeding. In addition, boosting growth performances is the result of enhancing digestive enzymes and appetite. Amylase, lipase, pepsin activities in stomach, and pepsin activity in intestine were dose-dependently increased in rainbow trout fed diet supplemented with 1% and 2% of lupin (*Lupinus perennis*), mango (*Mangifera indica*) and stinging nettle (*Urtica dioica*) in 2 months, following the increments of WG and SGR in all of extract treatments (Awad *et al.*, 2012). Three herbs (i.e. *Alteranthera sessilis*, *Eclipta alba*, and *Cissus quadrangularis*) at 0.25% functioned as appetizers and enhanced the activities of digestive enzymes (protease, amylase and lipase) of freshwater prawns after 90 days of feeding (Radhakrishnan *et al.*, 2014). A similar study of Giri *et al.* (2017) also revealed that 3% of *Hybanthus enneaspermus* (Linn F. Muell.) aqueous extract-based diet significantly enhanced the activities of intestinal digestive enzymes (amylase, protease, and lipase), and then stimulated the increase of final weight and SGR in rohu *Labeo rohita* after 6 weeks. Extract-based diet could improve lipid metabolism and modulate the activities of trypsin, an important enzyme of the digestive processes, resulting in an increase in feed conversion and leading to higher protein synthesis (Bulfon *et al.*, 2015). After 15 days of feeding, the total lipid decreased in line with the increase of total protein in Asian sea bass (*Lates calcarifer* (Bloch) fed with 0.3, 0.5, 1% ginger (*Zingiber officinale* Roscoe) extract enriched diets (Talpur *et al.*, 2013). These authors also found that weight gain, growth and feed conversion were significantly enhanced in those fish fed ginger diets. Similarly, growth parameters, feed conversion and protein efficiency significantly increased after 14 days in rainbow trout fed diets supplemented with 1% of garlic (*Allium sativum*) (Singh and Lakra, 2012).

Indeed, dietary supplemented plant products did not always show positive effects in enhancement of fish growth. A reduction of growth performance and nutrient utilization has been recorded in Nile tilapia fed diet containing over 5% of alfalfa meal for 67 days (Ali *et al.*, 2003). Similarly, Nile tilapia showed reduction in growth rate after feeding diet containing extracts of moringa for 10 days (Dongmeza *et al.*, 2006).

6.3.2. Plant extracts act as immunostimulants

Many medicinal plants have been reported to be effective in fish and shellfish as feed additives. The use of plant products is mainly for boosting the immune and defence mechanisms conferring the protection of animals from infectious diseases. The use of plants has mostly gained ground for various reasons such as availability, environmental and animal compatibility, spontaneous decomposition, and the possibility of production in large quantities at a low price in aquatic animals (Pandey *et al.*, 2012). Due to the presence of the various compounds and secondary metabolites, plant products display immunomodulatory activities on lysozyme, complement, antiprotease, myeloperoxidase, reactive oxygen species, reactive nitrogen species, phagocytosis, respiratory burst activity, nitric oxide, blood parameters and total immunoglobulin (Table 1).

Powerful effects of olive extracts were demonstrated in common carp where using 0.1% showed significant improvement in most of the tested immune parameters including hematocrit ratio, serum myeloperoxidase activity, immune response gene levels (*il1β* in head kidney tissue and *tnfα* in spleen tissue). Moreover, the survival rate in common carp was significantly improved after feeding with olive extracts (Zemheri-Navruz *et al.*, 2019).

Hematological and serum biochemical parameters are widely used in order to determine the effects of feed additives on fish health. In common carp and Nile tilapia, the blood indices were significantly increased after feeding with *E. hirta*-based diets (Pratheepa and Sukumaran, 2011, Pratheepa and Sukumaran, 2014), *P. guajava* and *M. dubia* extract-based diets (Yunis-Aguinaga *et al.*, 2016, Omitoyin *et al.*, 2019). Similar results were obtained in Ningu fed *C. limon* (Ngugi *et al.*, 2017), rainbow trout fed *C. sativum* (Naderi Farsani *et al.*, 2019), and ruho fed *H. enneaspermus* (Giri *et al.*, 2017).

Measurement of lysozyme activity is a way to determine whether non-specific immune responses are boosted by immunostimulants. Lysozyme is primarily released by monocytes and found to be higher in macrophages (Ogundele, 1998). In addition, lysozyme possesses both bactericidal and opsonin effects that result in the activation of the complement system and phagocytes to prevent infectious diseases (Ogundele, 1998). Besides the non-specific humoral immune system, total immunoglobulin also plays an essential role in host defence mechanisms and acts as a biomarker for fish adaptive immunity (Chen *et al.*, 2013). Most of the studies have demonstrated that plant extract-based diets positively affected to humoral innate immune (lysozyme and complement activity) and adaptive immune (total Ig) responses in aquatic animals after feeding with plant product-based diets (Table 1). Moreover, the skin is the first physical barrier against the invasion of environmental pathogens in fish (Esteban, 2012). Skin mucus possesses various humoral immune parameters (i.e., complement proteins, immunoglobulins, lectins, lysozyme, proteases, and antimicrobial peptides) (Lazado and Caipang, 2014). Several studies also demonstrated that skin mucus immune parameters (i.e. total Ig, lysozyme, peroxidase, and antibacterial) increased in rohu fed *Z. officinale* (Sukumaran *et al.*, 2016), in rainbow trout fed *M. communis* (Mansouri Taee *et al.*, 2017), in

common carp fed *M. germanica* (Hoseinifar *et al.*, 2017), and in Nile tilapia fed *C. sinensis* (Van Doan *et al.*, 2019).

Phagocytosis is one of the first steps in the stimulation of the immune response and inflammation (Vatansever *et al.*, 2013). In invasive pathogens, reactive oxygen (ROS) and reactive nitrogen (Corripio-Miyar *et al.*) production are primarily released throughout the phagocytic process (Magor and Magor, 2001, Chakrabarti *et al.*, 2014). Phagocytic cells are the most important cellular components of the innate immune system of fish (Macarthur and Fletcher, 1985). Phagocytes also produce toxic oxygen forms during the process called respiratory burst (Neumann *et al.*, 2001), which is also important for the fish immune system (Seeley *et al.*, 1990). Administration of plant products enhanced the phagocytic, respiratory burst activity, reactive oxygen and reactive nitrogen production in various fish species. Indeed, the phagocytosis and respiratory burst activity of leukocytes were significantly promoted in Asian seabass fed *A. indica* (Talpur and Ikhwanuddin, 2013) and *M. piperita* (Talpur, 2014), in common carp fed *E. hirta* (Pratheepa and Sukumaran, 2011, Pratheepa and Sukumaran, 2014), in cobia fed *P. asiatica* and *M. haplocalyx* (Wu *et al.*, 2016), in gilthead sea bream and European sea bass fed *C. coggygria* and *M. sylvestris* (Bilen *et al.*, 2019), in ningu fed *C. limon* (Ngugi *et al.*, 2017), in rainbow trout fed *C. spinosa* (Bilen *et al.*, 2016) and *N. sativa* (Celik Altunoglu *et al.*, 2017), and in rohu fed *H. enneaspermus* (Giri *et al.*, 2017). Moreover, *R. glutinosa* and *P. guajava* extract-based diets significantly enhanced the phagocytosis activity in common carp and rohu leukocytes, respectively (Wang *et al.*, 2015, Giri *et al.*, 2015). An increase of respiratory burst activity was observed in bluga fed *A. cepa* (Akrami *et al.*, 2015), Nile tilapia fed *M. dubia* (Yunis-Aguinaga *et al.*, 2016), and rohu fed *M. indica* (Sahu *et al.*, 2007). Oral administration of *P. guajava* extract also significantly enhanced the ROS and NOS production in Nile tilapia (Gobi *et al.*, 2016). Also, Nile tilapia were intraperitoneally injected with *S. trilobatum* induced a significant stimulation of ROS and NOS production (Divyagnaneswari *et al.*, 2007).

Apart from the benefit of the improvement in the immune variables, the effects of plant extracts on several cytokine encoding genes (i.e. *tnfα*, *il8*, *il1β*, and *il10*) were also investigated as a valuable tool in aquaculture. IL-1β is recognized as an indicator of innate immunity and body response to various microbial agents and toxins (Secombes, 1996), which can stimulate immune responses via producing blood monocytes and tissue macrophages (Corripio-Miyar *et al.*, 2007). TNF-α is cell-inflammatory cytokines related to cell proliferation, apoptosis, leukocyte migration, phagocytic activity and other proinflammatory cytokines (Yuan *et al.*, 2008). IL-8 is produced by a number of cell types, such as macrophages/monocytes, epithelial cells, neutrophils, fibroblasts, and endothelial cells, which exhibit a strong inflammatory response to neutrophils and other leukocytes to the site of infection, or which is a chemokine induced by IL-1 and TNF-α (Jimenez *et al.*, 2006). The expressions of cytokine encoding genes such as *tnfα*, *il8*, and *il1β* were observed to increase in blunt snout bream fed Fenugreek seed (Yu *et al.*, 2019), in common carp fed Olea (Zemheri-Navruz *et al.*, 2019), *M. germanica* (Hoseinifar *et al.*, 2017) and *P. guajava* (Hoseinifar *et al.*, 2019b), in rainbow trout fed *C. spinosa* (Bilen *et al.*, 2016) and *N. sativa* (Celik Altunoglu *et al.*, 2017), and in rohu fed *Z.*

officinale. In addition, expressions of genes including *il10*, *tgfβ*, *inos*, and *lysozyme* were also regulated in fish fed plant product-based diets (Wang *et al.*, 2015, Celik Altunoglu *et al.*, 2017, Yu *et al.*, 2019).

Up to now, most studies have investigated the effects of dietary herbal extracts on some indicators of the immune response in different fish species. Vietnam has plenty of wild plant resources distributed in the different eco-regions (De Queiroz *et al.*, 2013). However, the use of natural products in aquaculture, as well as striped catfish culture, is not yet popularly investigated in the country. Only a study of Prabu *et al.* (2016) revealed that fucoidan rich seaweed (*Sargassum wightii*) extract-based diets enhanced the non-specific immune parameters and disease resistance against *A. hydrophila* in *P. hypophthalmus* after 45 days of feeding. Indeed, respiratory burst activity, lysozyme activity, phagocytic activity and total leukocyte count were increased with the increasing of extract concentration (1%, 2%, 3% seaweed extract), whereas serum Albumin/Globulin (A/G) ratio and blood glucose level exhibited decreasing trend. The expression of interferon gamma (*ifnγ*) gene in extract groups was higher than the control group in pre and post *A. hydrophila* infection.

Table 3. Medicinal plants administered through different routes at various concentrations enhanced growth and immune response of fish

Fish	Plants	Solvent / Parts	Doses (%)	Administration/ Duration	Immune parameters	Pathogens	Growth promotion	References
Asian seabass (*Lates calcarifer*)	Neem (*Azadirachta indica*)	Powder/leaves	0.1; 0.2; 0.3; 0.4; 0.5	Oral/2 weeks	Phagocytic, respiratory burst, lysozyme, bactericidal, antiprotease	*Vibrio harveyi*	+	(Talpur and Ikhwanuddin, 2013)
Asian seabass (*Lates calcarifer*)	Peppermint (*Mentha piperita*)	Leaves	0.1; 0.2; 0.3; 0.4; 0.5	Oral/4 weeks	Phagocytic, respiratory burst, lysozyme, bactericidal, antiprotease	*V. harveyi*	+	(Talpur, 2014)
Blunt snout bream (*Megalobrama amblycephala*)	Fenugreek seed	NA	0.04; 0.08; 0.16	Oral/8 weeks	Plasma complement, immunoglobulin, cytokine genes (*tnfα*, *il8*, *il10*) expression	NA	NA	(Yu *et al.*, 2019)
Bluga (*Huso huso* Linnaeus, 1754)	Onion (*Allium cepa*)	Commercial powder/ bulbs	0.5; 1	Oral/8 weeks	Lysozyme, respiratory burst, total protein, globulin, immunoglobulin	NA	+	(Akrami *et al.*, 2015)
Common carp (*Cyprinus carpio*)	Jujube (*Ziziphus jujube*)	Fruit	0.25; 0.5; 1	Oral/8 weeks	Total Ig, lysozyme and protease activity, cytokines genes (*il1b*, *il8*, *il10*, *tnfα*) expression	NA	+	(Hoseinifar *et al.*, 2019a)
Common carp (*Cyprinus carpio*)	Olive (*Olea* europea L.)	70% ethanol/ leaves	0.1; 0.25; 0.50; 1	Oral/60 days	Hematocrit ratio, serum myeloperoxidase activity, immune response genes (*il1β*, *il8*, *tnfα*) expression	*Edwardsiella tarda*	NA	(Zemheri-Navruz *et al.*, 2019)
Common carp (*Cyprinus carpio*)	Asthma plant (*Euphorbia hirta*)	leaves	0.5; 1; 2; 2.5; 5	Oral/50 days	Red blood cells, white blood cells, lysozyme, phagocytic, NBT assays	*A. hydrophila*	NA	(Pratheepa and Sukumaran, 2014)
Common carp (*Cyprinus carpio*)	Asthma plant (*Euphorbia hirta*)	leaves	0.5; 1; 2; 2.5; 5	Oral/50 days	Red blood cells, white blood cells, lysozyme, phagocytic, NBT assays	*Pseudomonas fluorescens*	NA	(Pratheepa and Sukumaran, 2011)

Table 3. Medicinal plants administered through different routes at various concentrations enhanced growth and immune response of fish

Fish	Plants	Solvent / Parts	Doses (%)	Administration/ Duration	Immune parameters	Pathogens	Growth promotion	References
Common carp (*Cyprinus carpio*)	*Rehmannia glutinosa*	Aqueous/ root	2; 4	Oral/60 days	Lysozyme, phagocytic, immune gene (*il1β*, *inos*, *tnfα*)	*A. hydrophila*	+	(Wang *et al.*, 2015)
Common carp (*Cyprinus carpio*)	Medlar (*Mespilus germanica*)	80% ethanol/ leaves	0.25; 0.50; 1	Oral/49 days	Skin mucus total Immunoglobulin, lysozyme, some genes (*il1β*, *il8*, *tnfα*, *tgfβ*) expression	NA	+	(Hoseinifar *et al.*, 2017)
Common carp (*Cyprinus carpio*)	Dill (*Anethum graveolens*) and Garden cress (*Lepidium sativum*)	40% Methanol/ Whole plant	0.1; 0.2	Oral/45 days	Lysozyme, Myeloperoxidase	*A. hydrophila* and *E. tarda*	+	(Bilen *et al.*, 2018)
Common carp (*Cyprinus carpio*)	Guava (*Psidium guajava*)	crude powder/ leaves	0.25; 0.5; 1	Oral/8 weeks	Total Ig, alkaline phosphatase activity, lysozyme activity, some genes (*il1β*, *il8*, *tnfα*) expression	NA	NA	(Hoseinifar *et al.*, 2019b)
Common carp (*Cyprinus carpio*)	Henna (*Lawsonia inermi*)	80% methanol/ leaves	6, 60 or 600 mg/kg body weight	Intraperitoneal injected/ 10 days	Lysozyme and bactericidal activity, phagocytic and respiratory burst activity	*A. hydrophila*		(Soltanian and Fereidouni, 2016)
Cobia (*Rachycentron canadum*)	Plantain (*Plantago asiatica*), fishwort (*Houttuynia cordata*), Mentha (*Mentha haplocalyx*)		0.25; 0.5; 1; 2	Oral/6 weeks	Phagocytic, respiratory burst, lysozyme	NA	+	(Wu *et al.*, 2016)

Table 3. Medicinal plants administered through different routes at various concentrations enhanced growth and immune response of fish

Fish	Plants	Solvent / Parts	Doses (%)	Administration/ Duration	Immune parameters	Pathogens	Growth promotion	References
Gilthead sea bream (*Sparus aurata*)	Tassel (*Muscari comosum*)	bulbs	0.5; 2 mg/kg body weight	Intraperitoneal injected/ 28 days	Respiratory burst, lysozyme, total protein,	NA	+	(Baba *et al.*, 2014)
Gilthead sea bream (*Sparus aurata*), European Sea bass (*Dicentrarchus labrax*)	Tetra (*Cotinus coggygria*) and Common mallow (*Malva sylvestris*)	40% methanol	0.05; 0.1; 1	Oral/60 days	Respiratory burst, phagocytic, lysozyme, myeloperoxidase and bacterial killing activities	*Vibrio anguillarum*	+	(Bilen *et al.*, 2019)
Golden pompano (*Trachinotus ovatus*)	Dandelion (*Taraxacum officinale*)	Root powder	0.05; 0.1; 0.2; 0.3; 0.4; 1	Oral/8 weeks	Plasma lysozyme complement 3, complement 4, immunoglobulin M	*V. harveyi*	+	(Tan *et al.*, 2017)
Ningu (*Labeo victorianus*)	Bitter lemon (*Citrus limon*)	Essential oils	1; 2; 5; 8	Oral/28 days	Red blood cells, white blood cells, lymphocytes, neutrophils, phagocytic, serum lysozyme, total immunoglobulin, respiratory burst activity	*A. hydrophila*	+	(Ngugi *et al.*, 2017)
Nile tilapia (*Oreochromis niloticus*)	Assam tea (*Camellia sinensis*)	Leaves/ethanol	0.1; 0.2; 0.4; 0.8	Oral/4 and 8 weeks	Serum and mucosal lysozyme, peroxidase, alternative complement, phagocytosis, and respiratory burst	*Streptococcus agalactiae*	+	(Van Doan *et al.*, 2019)

Table 3. Medicinal plants administered through different routes at various concentrations enhanced growth and immune response of fish

Fish	Plants	Solvent / Parts	Doses (%)	Administration/ Duration	Immune parameters	Pathogens	Growth promotion	References
Nile tilapia (*Oreochromis niloticus*)	Guava (*Psidium guajava* L.)	Aqueous/leaves	0.25; 0.50; 0.75; 1	Oral/84 days	Red blood cells, White blood cells, monocytes, lymphocytes, eosinophils, basophils	*A. hydrophila*	+	(Omitoyin *et al.*, 2019)
Nile tilapia (*Oreochromis mossambicus*)	Guava (*Psidium guajava* L.)	Ethanol/ leaves	0.1; 0.5; 1	Oral/30 days	Myeloperoxidase, reactive oxygen and reactive nitrogen, complement, lysozyme	*A. hydrophila*	+	(Gobi *et al.*, 2016)
Nile tilapia (*Oreochromis mossambicus*)	Camu camu (*Myrciaria dubia*)	Commercial extract	0.005; 0.01; 0.025; 0.05	Oral/5 weeks	White blood cells counts in blood and exudate, burst respiratory, lysozyme, serum bactericidal	*A. hydrophila*	NA	(Yunis-Aguinaga *et al.*, 2016)
Nile tilapia (*Oreochromis mossambicus*)	Solanum (*Solanum trilobatum*)	Methanol/ leaves	4; 40; 400 mg/kg body weight	Intraperitoneal injected/ 10 days	Lysozyme, reactive oxygen species, reactive nitrogen species	*A. hydrophila*	NA	(Divyagnaneswari *et al.*, 2007)
Nile tilapia (*Oreochromis mossambicus*)	Heartleaf moonseed (*Tinospora cordifolia*)	Water-soluble fraction/ leaves	6, 60 or 600 mg/kg body weight	Intraperitoneal injected/ 10 days	Serum lysozyme, antiprotease and natural hemolytic complement activities, reactive oxygen and reactive nitrogen production	*A. hydrophila*	NA	(Alexander *et al.*, 2010a)
Zebrafish (*Danio rerio*)	Myrtle (*Myrtus communis* L., Myrtaceae)	Aerial parts powder	0.5; 1; 2	Oral/60 days	Lysozyme, total Ig and protease, some genes (*lyz*, *tnfα*) expression	NA	+	(Safari *et al.*, 2017)
Rainbow trout (*Oncorhynchus mykiss*)	Myrtle (*Myrtus communis* L., Myrtaceae)	Aerial parts powder	0.5; 1; 1.5	Oral/60 days	Skin mucus lysozyme, antimicrobial activity,	NA	+	(Mansouri Taee *et al.*, 2017)

Table 3. Medicinal plants administered through different routes at various concentrations enhanced growth and immune response of fish

Fish	Plants	Solvent / Parts	Doses (%)	Administration/ Duration	Immune parameters	Pathogens	Growth promotion	References
Rainbow trout (*Oncorhynchus mykiss*)	Coriander (*Coriandrum sativum*)	80% methanol/seed	0.5; 1; 2	Oral/8 weeks	Red blood cells, White blood cells, lysozyme, alternative complement activity, total immunoglobulin	*Yersinia ruckeri*	+	(Naderi Farsani *et al.*, 2019)
Rainbow trout (*Oncorhynchus mykiss*)	Caper (*Capparis spinosa*)	40% Methanol	0.01; 0.05	Oral/30 days	Phagocytic, lysozyme, myeloperoxidase, respiratory burst cytokine genes (*il1β*, *il8*, *tnfα*, *il10* and *il12*) expression	*A. hydrophila*	NA	(Bilen *et al.*, 2016)
Rainbow trout (*Oncorhynchus mykiss*)	Black cumin (*Nigella sativa*)	Methanol/seeds	0.01; 0.05	Oral/30 days	Respiratory burst, lysozyme, myeloperoxidase, phagocytic, some genes (*il1β*, *il8*, *il12p40*, *tnfα1*, *tgfβ*, *il10*) expression	*A. hydrophila*	+	(Celik Altunoglu *et al.*, 2017)
Rainbow trout (*Oncorhynchus mykiss*)	Hala (*Pandanus tectorius*)	95% alcohol/ leaves	0.5; 1; 2	Oral/2 weeks	Total protein, myeloperoxidase content, antiproteases, lysozyme and bactericidal activities, some genes (*tnf*, *lyz2*, *il8* and *cd4*) expression	*Yersinia ruckeri*	NA	(Awad *et al.*, 2019)
Rohu (*Labeo rohita*)	Mango (*Magnifera indica*)	kernel	0.1; 0.5; 1	Oral/60 days	Respiratory burst, lysozyme, serum bactericidal, total protein	*A. hydrophila*	NA	(Sahu *et al.*, 2007)

Table 3. Medicinal plants administered through different routes at various concentrations enhanced growth and immune response of fish

Fish	Plants	Solvent / Parts	Doses (%)	Administration/ Duration	Immune parameters	Pathogens	Growth promotion	References
Rohu (*Labeo rohita*)	Spade Flower (*Hybanthus enneaspermus* Linn F. Muell.)	Whole plants/ aqueous	0.1; 0.2; 0.3; 0.4	Oral/6 weeks	Red blood cells, White blood cells, monocytes, lymphocytes, eosinophils, basophils, phagocytic, serum lysozyme, alternative complement pathway, respiratory burst	*A. hydrophila*	+	(Giri *et al.*, 2017)
Rohu (*Labeo rohita*)	Ginger (*Zingiber officinale*)	Rhizome powder	0.2; 0.4; 0.6; 0.8; 1	Oral/30 days and 60 days	Skin mucus lysozyme, immunoglobulin, anti-bacterial activity, some genes (*il10*, *il1β*, *tnfα*) expression	*A. hydrophila*	+	(Sukumaran *et al.*, 2016)
Rohu (*Labeo rohita*)	Guava (*Psidium guajava* L.)	Leaf powder	0.1; 0.5; 1; 1.5	Oral/60 days	Lysozyme, leukocyte phagocytic, and alternative complement pathway activity, IgM, some genes (*il1β*, *il10*, *inos*, *tnfα*, *cox2*, *nfκb*) expression	*A. hydrophila*	+	(Giri *et al.*, 2015)

NA: Not available, *IL1β*: interleukin 1β; *IL10*: interleukin 10; *INOS*: Inducible nitric oxide synthase; *TNFα*: Tumor necrosis factor; *COX2*: Cyclooxygenase-2; *NFκB*: nuclear factor kappa-light-chain-enhancer of activated B cells; *LYZ*: lysozyme;

6.3.3. Plant extracts act as fish anti-pathogenic agents

As an overall consequence of immunomodulatory effects, certain medicinal plants could improve the resistance to infectious diseases in fish. Diets supplemented with *A. indica* (Talpur and Ikhwanuddin, 2013) and *M. piperita* (Talpur, 2014) were reported to significantly ameliorate the immune responses and the survival rate of Asian seabass against *V. harveyi* after 2 and 4 weeks of feeding, respectively. Similarly, the mortality in common carp was significantly improved following *E. hirta*-based diets against *A. hydrophila* and *P. flourescens* (Pratheepa and Sukumaran, 2011, Pratheepa and Sukumaran, 2014); Ol*ea*-based diets against *E. tarda* (Zemheri-Navruz *et al.*, 2019); *R. glutinosa*-based diets against *A. hydrophila* (Wang *et al.*, 2015); and *A. graveolens* and *L. sativum*-based diets against *A. hydrophila* and *E. tarda* (Bilen *et al.*, 2018). Oral administration of *P. guajava* extract (Gobi *et al.*, 2016, Omitoyin *et al.*, 2019) or *M. dubia* extract (Yunis-Aguinaga *et al.*, 2016) increased the resistance of Nile tilapia to *A. hydrophila* via enhancing blood parameters, lysozyme, complement, respiratory burst, serum bactericidal. Also, Nile tilapia fed *C. sinensis*-based diets significantly improved mortality against *S. agalactiae* (Van Doan *et al.*, 2019). Moreover, the intraperitoneal injection of *S. trilobatum* reduced the susceptibility of Nile tilapia to *A. hydrophila* due to the improvement of lysozyme, reactive oxygen species and reactive nitrogen species (Divyagnaneswari *et al.*, 2007). Diets enriched with *C. sativum* extract (Naderi Farsani *et al.*, 2019) and *P. tectorius* extract (Awad *et al.*, 2019) significantly reduced the mortality in Rainbow trout against *Y. ruckeri*. Similarly, *C. spinosa* (Bilen *et al.*, 2016) and *N. sativa* (Celik Altunoglu *et al.*, 2017) extract-based diets could stimulate the disease resistance of Rainbow trout to *A. hydrophila.* Long-term dietary administration of *M. indica* (Sahu *et al.*, 2007), *P. guajava* (Giri *et al.*, 2015), *Z. officinale* (Sukumaran *et al.*, 2016), and *H. enneaspermus* (Giri *et al.*, 2017) strongly prevented the *A. hydorphila* infection in Rohu. In addition, several studies also demonstrated medicinal plants could protect the fish again pathogenic infection (i.e. *C. coggygria* and *M. sylvestris*-based diets against *V. anguillarum* infection in Gilthead sea bream (Bilen *et al.*, 2019), *T. officinale*-based diets against *V. harveyi* infection in golden pompano (Tan *et al.*, 2017), *C. limon* oil-based diets against *A. hydrophila* infection in Ningu (Ngugi *et al.*, 2017).

Enhancing the immune responses leads to the increase in disease resistance in fish. Moreover, several medicinal plants also act as antipathogenic agents, which could directly lock or inhibit the growth of pathogens (i.e. bacteria, fungi, parasites, or viruses) in host cells thus reducing their replication. Indian almond extract was an alternative antibacterial remedy against the bacterial pathogen *A. hydrophila* in tilapia (Chitmanat *et al.*, 2003). *Rosmarinus officinalis* was used to treat *Streptococcus* infection in tilapia (*Oreochromis* sp.) (Abutbul *et al.*, 2004). Several plants possess the ability to inhibit or block the transcription of the virus to reduce the replication in the host cells, and enhance innate immunity (Citarasu, 2010). Intraperitoneal administration of *Punica granatum* extracts increased disease resistance against lymphocystis disease virus in Olive flounder (*Paralichthys olivaceus*) (Harikrishnan *et al.*, 2010). On the other hand, ethanol extracts of *Piper guineese* (fruits) and *Xylopia aethiopica* (seeds) were

active against the fungus *Candida albicans* and bacteria *B. subtilis*, *Escherichia coli*, *S. typhi* (Okeke *et al.*, 2001).

6.3.4. Plant extracts act as vaccine adjuvant

Using immunostimulant as an adjuvant of fish vaccine is an attractive method for improving the protective capabilities of fish (Aly *et al.*, 2016). Oral administration with Miers (Guduchi) leaf extract (*Tinospora cordifolia*) two days before being vaccinated with heat-killed *A. hydrophila* increased a prolonged antibody response and disease resistance in Mozambique tilapia to *A. hydrophila* (Sudhakaran *et al.*, 2006). Innate immune parameters (i.e. lysozyme, total protein, superoxide dismutase, phagocytosis and nitric oxide), as well as adaptive responses (i.e. specific IgM), were increased in murrel fresh water fish (*Channa punctatus*) fed with 5% of banyan (*Ficus benghalensis*) root after immunized intramuscularly with bovine serum albumin (Verma *et al.*, 2012). The vaccinated fish using the *Aloe vera* adjuvant significantly produced antibody titers and leucocyte numbers, leading to increased relative percent survival when fish were challenged with *A. hydrophila* 28 days later (Zanuzzo *et al.*, 2015). A study of Thangamani *et al.* (2014) also revealed that Mozambique tilapia injected with chaff-flower (*Achyranthes aspera*) extract two days before *A. hydrophila* vaccination significantly increased the antibody response at lower doses (0.0002, 0.002, 0.02, 0.2, and 2 mg/fish), while a suppressive effect was observed at the highest dose (20 mg/fish) at 25 days post vaccination. Similarly, grey mangrove extract (*Avicennia marina*)-based diets at 1, 2 and 4% for 8 weeks after vaccination with living *V. alginolyticus* enhanced respiratory burst, phagocytic, lysozyme and complement activities, as well as the survival rate in clownfish (*Amphiprion sebae*) (Dhayanithi *et al.*, 2015). Mozambique tilapia were intraperitoneally injected with methanol extract of Parijat (*Nyctanthes arbortristis*) and then immunized with heat-killed *A. hydrophila* displayed an increase of antibody titers, lysozyme, myeloperoxidase activity, leucocyte count as well as an enhanced survival rate (Kirubakaran *et al.*, 2016)

7. Factors influencing the effectiveness of plant products

7.1. Route of extract administration

Medicinal herbs have several advantages such as easy preparation, low cost, action against a broad range of pathogens and direct effects on immune cells (Van Hai, 2015). However, the effects are variable among fish species and depend mainly on different factors like dosage, time, route of administration of the compounds and the general physiological conditions of the fish (Gannam and Schrock, 1999, Harikrishnan *et al.*, 2011a).

Most of the plant extracts are often administered orally, which is the best way for stimulating target animal immune functions, and less frequently via injection or immersion for preventive purposes or disease treatment. Oral administration is the most practical and preferable method that is suitable for extensive aquaculture. It is a non-stressful method and permits the larger number of fish in all sizes to be treated with minimum cost and effort (Sakai, 1999, Galindo-Villegas and Hosokawa, 2004, Selvaraj *et al.*, 2005). Many authors have demonstrated that oral administration of medicinal plants and their derived products resulted in enhancing the innate

immune responses and the resistance to infectious pathogens (Reverter *et al.*, 2014, Harikrishnan *et al.*, 2011a, Awad and Awaad, 2017). Although oral delivery of immunostimulants, as well as plant products, is the effective and the most suitable method for fish farming, it is slowly absorbed by the fish (Yoshida *et al.*, 1995). Administration by injection has been demonstrated as a fast and very effective method, but this is an invasive and laborious method, and it is only applied for large fish higher than 15 g (Duncan and Klesius, 1996, Yoshida *et al.*, 1995). Few studies revealed that the injection with plant extracts significantly increased the fish humoral and cellular immune response, as well as improved the survival after bacterial infection (Wu *et al.*, 2010, Alexander *et al.*, 2010b). Although intraperitoneal injection is a very effective method, it look relatively time-consuming and becomes impractical in case of fish less than 15 g (Harikrishnan *et al.*, 2011a). Several authors also have indicated that immersion acts as another effective method, which could enhance the immune responses in fish farming (Anderson *et al.*, 1995, Harikrishnan *et al.*, 2009a). Common carp immersed in 1 g/L of *A. indica* leaf extract (10 min/day for 30 days) increased the total protein and protected fish from *A. hydrophila* infection (Harikrishnan *et al.*, 2003). This method is more practical for small fish size although it requires preparation of a large quantity of dipping solution which could be costly (Awad and Awaad, 2017).

7.2. Dose and duration of administration

The immune capacity of plants is firstly dependent on the dosages used, which means that the optimum dose that triggers maximum immune response needs to be investigated. Common carp (*Cyprinus carpio*) fed diets supplemented with 0.25, 0.5 and 1% of jujube (*Ziziphus jujuba* Mill.) fruit extract showed the highest total Ig in skin mucus and the highest level of *il1b* expression at the dose of 0.5%, while doses at 0.5 and 1% significantly increased skin mucus protease activity and *il8* expression (Hoseinifar *et al.*, 2019a). Mozambique tilapia were injected with 4, 40 or 400 mg/kg of body weight of *Solanum trilobatum* extract showed enhancement in reactive oxygen species production only with the dose of 40 mg/kg (Divyagnaneswari *et al.*, 2007). Although assam tea (*Camellia sinensis*) extract-based diets at 0.1, 0.2, 0.4, 0.8% differentially enhanced the humoral and mucosal immune responses in Nile tilapia, the maximum significant immune responses were recorded at the dose of 0.2%. The highest survival rate was also observed in fish fed 0.2% assam tea extract after *S. agalactiae* infection (Van Doan *et al.*, 2019).

Moreover, the enhancement of immune response is also variable with the different types of medicinal plants and the cultivated species used. Single aqueous methanolic extracts of tetra (*Cotinus coggygria*) or common mallow (*Malva sylvestris*) at 0.5 and 1% were supplemented to gilthead sea bream and European sea bass diets. The phagocytic activity in sea bass was significantly increased in tetra extract-based diets, while both tetra and common mallow extract-based diets significantly increased the phagocytic activity in sea bream at 30 days post feeding. Similarly, lysozyme activity was enhanced in all tetra and common mallow groups in sea bass, and in only tetra groups in sea bream (Bilen *et al.*, 2019).

In addition, the effectiveness of plant products was influenced by the duration of the experiment. Most of the medicinal plants and their products were applied in fish with doses ranging from 0.005 to 8% for 2 to 60 days (table 1). Nile tilapia fed guava aqueous extracts at 0.1 and 1% did not display changes in the myeloperoxidase and reactive oxygen species activities enhanced at 10 and 20 days post feeding, while these activities were significantly increased after 30 days of feeding (Gobi *et al.*, 2016). Olive flounder fed diets supplemented with 0.1 and 1.0% of *Prunella vulgaris* extract recorded a significant increase in phagocytic activity at 1, 2 and 4 weeks, while the highest lysozyme activity was observed at 0.01, 0.1 and 1% on weeks 2 and 4 (Harikrishnan *et al.*, 2011b). Although *Eclipta alba*-based diets at 0.01, 0.1 and 1% enhanced the complement activity of Mozambique tilapia after 1 and 2 weeks post feeding compared to the control, this activity declined after 3 weeks (Christybapita *et al.*, 2007).

8. Conclusions

The striped catfish aquaculture in Vietnam has spectacularly developed as in over the past decade. However, the intensive culture of striped catfish has caused serious stress to culture environments and increased the incidence of infectious diseases, especially bacteria-induced diseases. With the purposes of prophylactic therapeutic, antibiotics and chemical agents has been overapplied in fish farming, which has led to many negative emergencies on the environments as well as human health. Thus, many attempts have been developed to resolve these issues. In recent years, immunostimulants including inactivated vaccination, probiotics, prebiotics, microbial derivative, and synthetic factors have been successfully applied in striped catfish aquaculture to significantly improve the fish's health status and reduce the occurrence of infectious diseases. Moreover, boosting the immune response using ecologically friendly compounds is an effective strategy to promote sustainable aquaculture. Previous research reports that plant extracts have been successfully applied to improve aquatic organisms' immunity and disease resistance. With the excellent potential of medicinal properties of plant products, our studies are next to focus on the development of a suitable method to replace traditional treatment in striped catfish culture.

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Chapter 2

Objective, scientific strategy and thesis outline

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1. Objective and scientific strategy

With the aims to significantly reduce the inappropriate use of antibiotics and other chemicals in aquaculture, the general objective of the present study aimed to identify some promising natural products via the evaluation and comparison of their effects on the immunomodulation as well as disease resistance in striped catfish.

Scientific strategies and research activities conducted during the thesis:

i. The best promising ethanol plant extracts were rapidly screened by evaluating the effects of different plant extracts on some innate and adaptive immune indicators (lysozyme, complement, and total immunoglobulin) using *in vitro* approaches based on striped catfish peripheral blood mononuclear cells (PBMC) and head kidney leukocytes (HKLs).

ii. Selected plant extracts displaying the highest immune responses were tested in striped catfish juveniles through an vivo experiment

iii. The efficiency of the most efficient plant extracts identified in the two first phases was compared when provided as single feed additive (at different dietary concentrations) or when supplied as mixture, using husbandry performances, immune responses and disease resistance.

iv. The mechanisms of action of these two selected plants, used as single or mixture additives, were investigated through a gel-free proteomic approach.

v. The immunomodulatory action of the selected plant extracts in striped catfish juveniles were compared with those of the main fractions and some pure compounds identified from these plants, using both *in vitro* and *in vivo* approaches.

2. Outlines of the Thesis

The research context and the results of this scientific strategy and activities conducted throughout the thesis are presented in the following chapters:

Chapter 1 General introduction

Chapter 2 Outline of the thesis

Chapter 3 Fish, facilities and general methodologies

Chapter 4 Screening of immuno-modulatory potential of different herbal plant extracts using striped catfish (*Pangasianodon hypophthalmus*) leukocyte-based *in vitro* tests

Chapter 5 Plant extract-based diets differently modulate immune responses and resistance to bacterial infection in striped catfish (*Pangasianodon hypophthalmus*)

Chapter 6 Single or combined dietary supply of *Psidium guajava* and *Phyllanthus amarus* extracts differentially modulate immune responses and liver proteome in striped catfish (*Pangasianodon hyphophthalmus*)

Chapter 7 Effects of crude ethanol extracts, their fractions and pure compounds from *Phyllanthus amarus* and *Psidium guajava* on immune responses of striped catfish (*Pagasianodon hypophthalmus*) head kidney leukocytes

Chapter 8 *Psidium guajava*- dichloromethane and ethyl acetate extract fractions early ameliorate striped catfish (*Pangasianodon hypophthalmus*) status via immune response, inflammatory, and apoptosis pathways

Chapter 9 General discussion, conclusions and perspectives

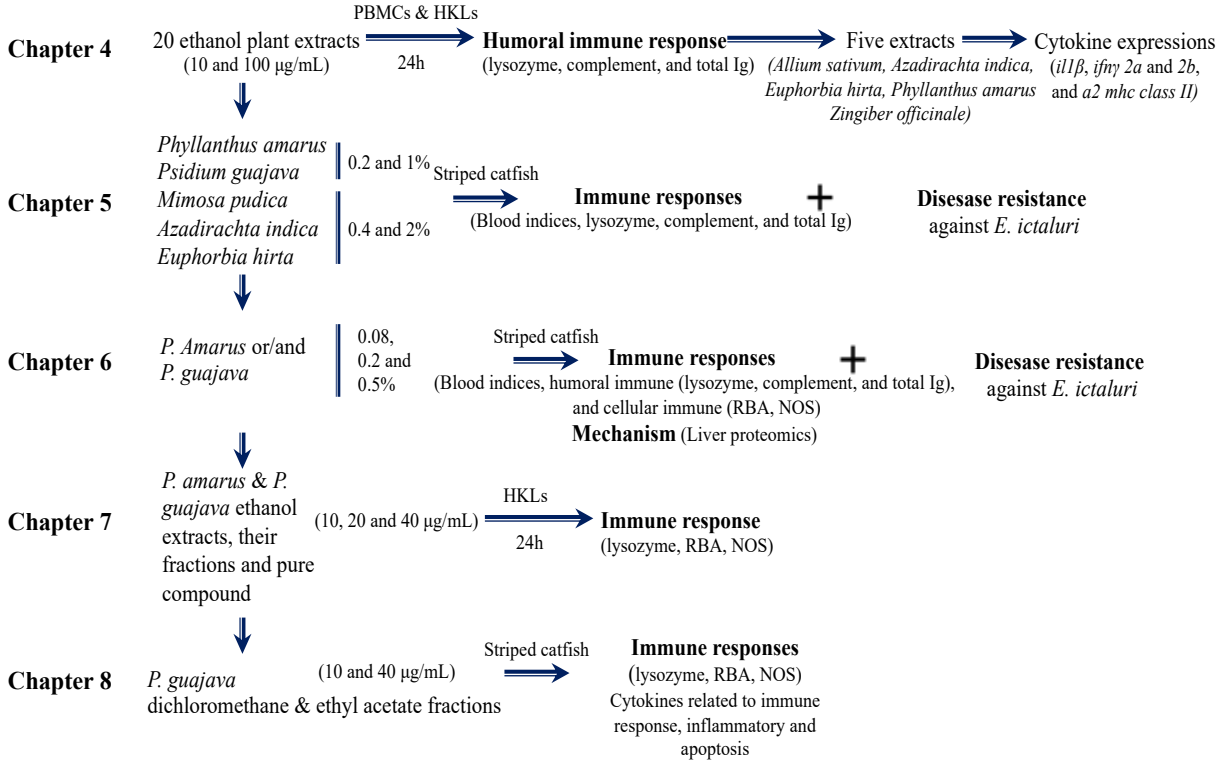

Figure 1. Summarizing of the main research activities in the thesis

Chapter 3

Fish, facilities and general methodologies

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1. Experimental fish

Striped catfish juveniles (50 ± 5 g for *in vitro*, 20 ± 5 g for *in vivo*) were obtained from a local fish farm in Vinh Long province, Vietnam, and transported to the laboratory in plastic bags filled with oxygenated water. The fish were acclimated to laboratory conditions for 15 days at 28±2°C in composite tank (2000 L). Fish were fed twice (9 am and 3 pm) daily at a feeding rate of 1% of body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco) under a natural photoperiod prior to their use in the *in vitro* and *in vivo* assay.

2. Isolation of PBMCs and HKLs

The isolation of PBMCs was performed according to the methods of Boyum (1968) modified by Pierrard *et al.* (2012). Briefly, blood was aseptically collected from the caudal vein with a sterile heparinized syringe. 2.5 mL of heparinized blood were quickly diluted with 4 mL of phosphate-buffered saline (PBS 1X); the mixture was then poured over a layer of 6 mL Ficoll Paque Plus (1.077 g mL^{-1}, GE Healthcare, Uppsala, Sweden) and centrifuged (400 g, 20min, 28±2°C). The white cells at the interface were collected and slowly washed twice with 5 mL cold sterile PBS 1X at low speed centrifugation (1000 g, 7min, 4°C).

Head kidney tissue was aseptically excised from freshly euthanized striped catfish and gently pushed through a 40-µm nylon mesh (VWR International, LLC, Radnor, PA USA) with L-15 medium (pH7.4, Sigma-Aldrich, St. Louis, MO, USA) supplemented with a 1% solution of 10,000 µg mL^{-1}streptomycin + 10,000 U mL^{-1} penicillin (Invitrogen).

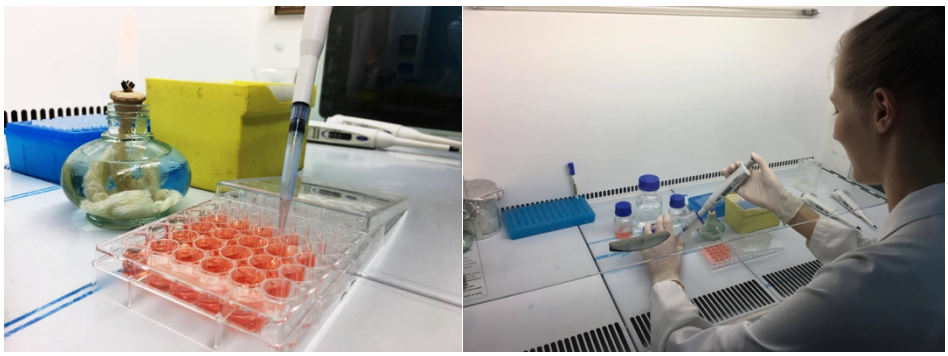

Figure 1. Peripheral blood mononuclear cells (PBMCs) and head kidney leukocytes (HKLs) stimulation by ethanol extracts

After washing, both PBMCs and HKLs were removed from residual erythrocytes by incubating them 5 min with an osmotic shock sterile red blood cell lysis buffer (pH 7.4). The suspension was neutralized by PBS 1X (v: v) and centrifuged as indicated previously, then the leukocytes were collected and suspended in L-15 medium supplemented with 5% fetal bovine serum

(FBS; Invitrogen), 1% Hepes (20 mM, Sigma, USA) and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A (PhA M form, Invitrogen).

Viable cells were adjusted to 5 x 10^6 cells mL^{-1} after enumeration using trypan blue stain (VWR, Leuven, Belgium) and seeded in wells of a 24 or 48-well plate for stimulated experiment (Greiner Bio-One, Vilvoorde, Belgium).

3. Extract preparation

Fresh plants were collected from Mekong Delta in Vietnam in separated experiments from March to June of 2016 (Chapter 4); from October to November of 2016 (Chapter 5), from April to May of 2017 (Chapter 7), and from May to June of 2016 (Chapter 8) . The plants were authenticated at the Department of Biology, College of Natural Science, Can Tho University. All collected parts of the plants were washed away from mud and dust; the rotten and damaged parts were discarded. The plants were air dried in shade for several days and then in oven at about 60^0C until well-dried. After that, they were grounded into fine powder by blender and stored in sealed containers in a dry and cool place.

Figure 2. Ethanol extract preparation for the experiments

The dried powder was soaked in ethanol 96% (volume ratio of dried powder: ethanol = 1: 20) for at least 24 h at room temperature with frequent agitation until the soluble matter had dissolved. The solvent-containing extracts were decanted and filtered. The ground samples were further extracted repeatedly four times with ethanol 96%. The filtrate from each extraction were combined and the solvent was evaporated under reduced pressure using a rotary evaporator to give crude ethanol extracts. All the well-dried crude ethanol extracts were stored at -20°C until used. The extracts were re-dissolved in dimethyl sulfoxide (DMSO, Saint Louis, MO, US) in order to prepare stock solution at 20 and 2 mg mL^{-1} and use in the following assays.

4. Diet preparation

The basal diet was prepared for two *in vivo* experiments (See Chapter 5 and Chapter 6, issue 2.2). The diet contained 30% crude protein, 6.66% crude lipid, 10.58% ash, 3.21% fibre, and 4.41 kcal/g energy. Fishmeal, soybean meal, cassava, and rice bran were mixed and sterilized at 110°C for 10 min (Mixture A). Butylated hydroxytoluene (BHT), vitamins and minerals

were well mixed with each of the plant extract concentrations (Mixture B). Then mixture A was mixed with mixture B and fish oil. The final mixture was extruded through a mini-extrusion machine (Can Tho University, Vietnam) at 70°C without steaming. Basal diets were supplemented with the different plant extracts at two concentrations for each plant extract as described above. The experimental feeds were then dried at 60°C for 24 h to achieve 11-12% moisture. Pellets of 2 mm were stored at –20°C until use.

Figure 3. Diet preparation for in vivo experiments

5. Bacteria preparation

E. ictaluri strain- Ed1 isolated from striped catfish in Mekong Delta of Vietnam were cultured on tryptic soy agar plates (Merck, MA, USA) for 48 h at 28°C following the method of Hang et al. (Hang *et al.*, 2013). Then, a single colony was collected and harvested into tryptic soy broth (Merck, MA, USA). This suspension was shaken overnight, 180 rpm at 28°C. Then, bacteria were centrifuged at 5000 rpm at 4°C for 5 min and washed 3 times with 0.85% NaCl solution. The mean colony count was found using the optical density method (Hoseinifar *et al.*) by spectrophotometer (Thermo spectronic, USA) at 590 nm, and OD value was adjusted to 0.1. This suspension was diluted 1000 times with NaCl solution and injected to the fish (Chapter 5 and Chapter 6).

Figure 4. Clinical signs of Striped catfish infected with *E. ictaluri* after 8 weeks of feeding extract-based diets.

6. Sample collection

The skin mucus samples were collected following the feeding trial (Chapter 5 and Chapter 6), and 3 days post injection (dpi) according to the method by Ross et al. (Ross *et al.*, 2000) with slight modification. Briefly, 3 fish per tank were randomly collected and anaesthetised using 0.1 ppm M222 (Sigma–Aldrich, MO, USA). Fish surfaces were individually washed with distilled water and then transferred into polyethylene bags containing 1 mL of PBS 1X. After 2 min of gentle shaking, mucus was collected, transferred to 2.0 mL sterile eppendorf tubes and centrifuged (1500 × *g* for 10 min at 4°C). The supernatant was stored at –80°C for further analysis. Similarly, blood samples were obtained from the caudal vein of individual fish (9 fish per treatment, 3 fish per tank) and centrifuged at 4000 rpm for 10 min. The plasma supernatant was collected into new eppendorf tubes and kept at –80°C until analysis.

Figure 5. Experiment system and sample collection in College of Aquaculture and Fisheries, CTU

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SECTION II

Chapter 4

Screening of immuno-modulatory potential of different herbal plant extracts using striped catfish (*Pangasianodon hypophthalmus*) leukocyte-based *in vitro* tests

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Nhu, T.Q., Hang, B.T.B., Vinikas, A., Hue, B.T.B., Quetin-Leclercq, J., Scippo, M.L., Phuong, N.T. and Kestemont, P.. Screening of immuno-modulatory potential of different herbal plant extracts using striped catfish (*Pangasianodon hypophthalmus*) leukocyte-based *in vitro* tests. Fish & shellfish immunology. 2019, 93, pp.296-307. DOI: 10.1016/j.fsi.2019.07.064.

Hypothesis outlines

To find a solution where plant products are available for the users, we firstly evaluated the modulation of 20 plant products, which potentially improved the immune system of striped catfish leukocytes. Lysozyme, complement and total immunoglobulin levels, as well as the expression of immune-related genes, have been commonly seen as valuable parameters in evaluating whether the plant products positively affect the fish immune system. The publications in peer reviewed international journals were regarded as the best available guarantee for data quality. The 20 plants were selected for their immunostimulatory, anti-oxidant and anti-microbial properties. Among the solvents, ethanol was demonstrated to yield more biological components after extraction, functioning in immunostimulatory properties and antibacterial activity. The ethanol is the best sovents because it is non toxic to cells or their constituents in cold extraction. Before the experiment, We also performed the prior test to find the optimal concentrations of several herbal extracts on the viability of peripheral blood mononuclear cells. The *A. indica* and *P. guajava* extracts at 150 and 200 µg/mL showed the high toxicity to the after 24 h stimulation. Therefore, the stimulated concentrations at 10 and 100 µg/mL were suggested for the *in vitro* screening test. Our *in vitro* tests will show how the ethanol plant extracts at two different concentrations regulate the immune responses of peripheral blood mononuclear cells and head kidney leukocytes of striped catfish. The effects of several plant extracts on two cell models will be also compared to deeply understand their responses at different time points. These questions will provide a better understanding of the capacity of plant extracts in activating the striped catfish leukocyte immune responses. Our first screening results will finally suggest the best plant extracts in stimulating the immune responses which will be applied for the next *in vivo* validation.

Abstract

Many medicinal plants have been shown to possess biological effects, including immuno-modulatory activities on human and other mammals. However, studies about the potential mechanisms of plant extracts on the humoral and tissular immunities in fish have received less attention. This study aimed to screen the immunestimulating properties of 20 ethanol plant extracts on striped catfish *Pangasianodon hypophthalmus* leukocytes. The peripheral blood mononuclear cells (PBMCs) and head kidney leukocytes (HKLs) of striped catfish (50 ± 5 gram per fish) were stimulated at 10 and 100 µg of each plant extract per mL of cell culture medium. Several humoral immune parameters (lysozyme, complement and total immunoglobulin) were examined at 24-hour post stimulation (hps). Furthermore, the responses of four cytokine genes, namely *il1β*, *ifrγ* 2a and b, and *mhc* class II were assessed by quantitative real-time PCR at 6, 12, 24, and 48 hps. The results showed that lysozyme, complement as well as total immunoglobulin levels in both PBMCs and HKLs were regulated by some of the plant extracts tested in a concentration-dependent manner; some plant extracts induced the highest immune responses at the low dose (10 µg mL^{-1}) while others were more efficient at high dose (100 µg mL^{-1}). Among the extracts, five extracts including garlic *Allium sativum* L. (As), neem *Azadirachta indica* A. Juss (Ai), asthma-plant *Euphorbia hirta* L. (Eh), bhumi amla *Phyllanthus amarus* Schum. et Thonn (Pa), and ginger *Zingiber officinale* Rosc (Zo) induced significant changes in the expression of pro-inflammatory cytokine (*il1β*), antiviral cytokines (*ifrγ* 2a and b) and adaptive immune cytokine (*mhc* class II) in striped catfish cells. Pa always modulated the strongest expression of the four cytokines in PBMCs and HKLs over the whole experimental period ($p < 0.05$), whereas Zo did not stimulate the *mhc* class II expression in striped catfish leukocytes throughout experimental periods. These *in vitro* results demonstrated that some plant extracts could differently modulate great potential immune response in fish, supporting their applications in further *in vivo* experiments.

1. Introduction

In fish, there is an interaction between innate and acquired immunity, although the adaptive immune response is more limited than that of higher vertebrates [1, 2]. It is well known that components of the immune response include humoral, cellular receptor molecules and central organs whose main function is involved in immune defense [3, 4]. The humoral immunity consists of lysozyme, hemolysin, immunoglobulins and complement molecules, whereas cellular immunity includes phagocytic cells, neutrophils, natural killer cells and lymphocytes [5-10]. Moreover, cytokines – including tumor necrosis factor- TNF-α, interferons- IFNs, and pro-inflammatory interleukins (*i.e.* IL-1β and IL-8) - also play an important role in the immune system. They could bind to specific receptors present in the cell membrane and lead to induction, enhancement or inhibition of a number of regulatory genes in the nucleus [11-13].

Striped catfish is one of the most important commercial fish species cultured in Vietnam in general, and Mekong Delta in particular. Like other fish species, striped catfish also suffers from many kinds of diseases including bacteria, fungi and parasites under stress conditions (*e.g.* poor water quality and/or high density of fish) [14, 15]. Such diseases have been

susceptible to cause enormous economic losses and reduced profit margins of the aquaculture sector [16]. In recent years, to resolve the mortality problem, many attempts have been made to strengthen striped catfish immune system. Administration of live or heat killed *Aeromonas hydrophila* by intraperitoneal injection strongly stimulated phagocytosis, respiratory burst, complement, lysozyme, plasma peroxidase, total IgM, and pathogen-specific antibody IgM [17]. Moreover, dietary supplementation with immunostimulants (*e.g. Escherichia coli* lipopolysaccharide, levamisole, and β-glucan) or probiotics (*e.g. Bacillus amyloliquefaciens* 54A and *Bacillus pumilus* 47B) significantly enhanced respiratory burst activity, lysozyme activity, complement activity, plasma anti-proteases, natural antibody titer and total protein. Furthermore, these compounds were also potentially involved in fish defense mechanism against *Edwardsiella ictaluri* infection [18-22].

Nowadays, herbal immunostimulants have been used with success as a more environmentally friendly approach in disease management. Herbal extracts are sources of many active components among which alkaloids, steroids, phenolics, tannins, terpenoids, saponins, and flavonoids that possess various biological activities [23-30]. These herbs have been supplied in fish diets to enhance the innate immune system, as general preventive measures in aquaculture [25, 31-38]. Dietary supplementation of Zo improved the non-specific immunity of Asian sea bass (*Lates calcarifer*) as showed by Talpur *et al*. [39]; whereas, Pratheepa and Sukumaran [40] found out an increase of phagocytic activity in common carp *Cyprinus carpio* proportionally to the dietary level of Eh. Gobi *et al.* [41] also proved that the ethanol leaf extract from Pg could play a major role in enhancing growth, antioxidant enzymes and immune variables in *Oreochromis mossambicus* compared with aqueous leaf extracts.

Up to now, most studies have investigated the effects of dietary herbal extracts on some indicators of the immune response in different fish species. However, there is limited information regarding the potential mechanisms of action of plant extracts on humoral and/or anti-inflammatory effects in fish immune cells. Sen *et al*. [42] reported that a flavonoid fraction of *P. guajava* leaf extract attenuated of LPS inducible *tnfα*, *il1β*, nitric oxide synthase- *inos* and cyclooxygenase- *cox*2 mRNA expression levels via the inhibition of nuclear factor kappa-light-chain-enhancer - NF-*κ*B of activated B cells in roho labeo (*Labeo rohita*) macrophages. Na-Phatthalung *et al.* [43] observed the induction of some cytokine genes (*il1β*, *il8*, *il10, tnfα* and *tgfβ*) on rainbow trout *Oncorhynchus mykiss* head kidney macrophages by stimulating with rose myrtle *Rhodomyrtus tomentosa* leaf extract and its active compound, rhodomyrtone. In this respect, *in vitro* approaches may provide interesting hypotheses on the potential mechanisms of the direct action of plant extracts (or their active compounds) on fish immune cells before their validation through *in vivo* experiments. Moreover, these tests can substantially reduce the time and the number of experimental animals for *in vivo* study as well as reduce the economic costs and the ethical problems.

Based on bibliography review data and on a survey in fish farms of Mekong Delta, 20 plants possessing potential immunostimulatory activities were selected for *in vitro* testing. In the present study, the immunomodulatory effects of ethanol plant extracts on the lysozyme and complement activities as well as on the total immunoglobulin in the striped catfish peripheral

blood mononuclear cells -PBMCs and head kidney leukocytes- HKLs were analyzed. After this first screening on 20 plant extracts, mRNA expression of cytokines (*il1β*, *ifnγ* 2a and 2b, *a2 mhc* class II integral membrane protein alpha chain 2- *mhc* class II) belonging to the innate and adaptive immunity was investigated at several time points after different stimulations with the five best extracts selected on the basis of the *in vitro* immune responses.

2. Material and methods

2.1. Extracts preparation

Twenty fresh plants were collected from Mekong Delta in Vietnam (Table 1). The plants were authenticated at the Department of Biology, College of Natural Science, Can Tho University. All collected parts of the plants were washed away from mud and dust; the rotten and damaged parts were discarded. The plants were air dried in shade for several days and then in oven at about 60^0C until well-dried. After that, they were grounded into fine powder by blender and stored in sealed containers in a dry and cool place.

Table 1: List of crude ethanol extracts

No	Family	Scientific name	Abbreviation	Part studied
1	*Asteraceae*	*Ageratum conyzoides* L.	Ac	Leaves, stem
2	*Meliaceae*	*Azadirachta indica* A. Juss	Ai	Leaves
3	*Annonaceae*	*Annona reticulate* L.	Ar	Leaves
4	*Alliaceae*	*Allium sativum* L.	As	Bulbs
5	*Amaranthaceae*	*Alternanthera sessilis* (L.) A. DC	Ase	Leaves, twigs
6	*Apiaceae*	*Centella asiatica* (L.) Urb	Ca	Whole plant
7	*Vitaceae*	*Cayratia trifolia* (L.) Domin	Ct	Leaves, stem
8	*Euphorbiaceae*	*Euphorbia hirta* L.	Eh	Leaves, twigs
9	*Asteraceae*	*Eclipta prostrata* (L.) L.	Ep	Leaves, twigs
10	*Saururaceae*	*Houttuynia cordata* Thunb.	Hc	Leaves, stem
11	*Cucurbitaceae*	*Momordica charantia* L.	Mc	Leaves
12	*Fabaceae*	*Mimosa pudica* L.	Mp	Leaves, twigs
13	*Lamiaceae*	*Ocimum basilicum* L.	Ob	Leaves, twigs
14	*Phyllanthaceae*	*Phyllanthus amarus* Schum. et Thonn.	Pa	Leaves, twigs
15	*Piperaceae*	*Piper betle* L.	Pb	Leaves
16	*Lamiaceae*	*Perilla frutescens* (L.) Britt	Pf	Leaves, twigs
17	*Myrtaceae*	*Psidium guajava* L.	Pg	Leaves
18	*Portulacaceae*	*Portulaca oleracea* L.	Po	Whole plant
19	*Compositae*	*Wedelia chinensis* (Osbeck) Merr	Wc	Whole plant
20	*Zingiberaceae*	*Zingiber officinale* Rosc.	Zo	Bulbs

The dried powder was soaked in ethanol 96% (volume ratio 1: 20) for at least 24 h at room temperature with frequent agitation until the soluble matter had dissolved. The solvent-containing extracts were decanted and filtered. The ground samples were further extracted repeatedly four times with ethanol 96%. The filtrate from each extraction were combined and the solvent was evaporated under reduced pressure using a rotary evaporator to give crude

ethanol extracts. All the well-dried crude ethanol extracts were stored at -20°C until used. The extracts were re-dissolved in dimethyl sulfoxide (DMSO, Saint Louis, MO, US) in order to prepare stock solution at 20 and 2 mg mL^{-1} and use in the following assays.

2.2. Experimental fish

Total 150 striped catfish juveniles (body weight = 50 ± 5 gram) were acclimated to laboratory conditions for 15 days at 28±2°C in composite tank (2000 L). Fish were fed twice (9am and 3pm) daily at a feeding rate of 1% of body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco) under a natural photoperiod prior to their use in the *in vitro* assay. The health status of experimental fish was checked following the method described by Biswas *et al* [44] with slight modifications. Briefly, few randomly sampled animals were examined for the presence of any abnormal lesions or parasites on body surfaces and internal organs [45]. Further, smears head kidney and blood from the same fish were cultured on tryptic soy agar plate (TSA, Merck) for 24-48 h at 28 °C for existence of any bacterial pathogens. Any colonies presented on TSA plate were used to perform PCR for detecting 16s RNA genes of commonly bacterial pathogens (*A. hydrophyla, E. ictaluri* and *Flavobacterium columnare*) in striped catfish [46]. Healthy fish which did not present any pathogenic bacteria, were used for experiment.

2.3. Isolation of PBMCs and HKLs

The isolation of PBMCs was performed according to the methods of Boyum [47] modified by Pierrard *et al.* [48]. Briefly, blood was aseptically collected from the caudal vein with a sterile heparinized syringe. 2.5 mL of heparinized blood were quickly diluted with 4 mL of phosphate-buffered saline (PBS 1X); the mixture was then poured over a layer of 6 mL Ficoll Paque Plus (1.077 g mL^{-1}, GE Healthcare, Uppsala, Sweden) and centrifuged (400 g, 20min, 28°C). The white cells at the interface were collected and slowly washed twice with 5 mL cold sterile PBS 1X at low speed centrifugation (1000 g, 7min, 4°C).

Head kidney tissue was aseptically excised from freshly euthanized striped catfish and gently pushed through a 40-µm nylon mesh (VWR International, LLC, Radnor, PA USA) with L-15 medium (pH7.4, Sigma-Aldrich, St. Louis, MO, USA) supplemented with a 1% solution of 10,000 µg mL^{-1}streptomycin + 10,000 U mL^{-1} penicillin (Invitrogen).

After washing, both PBMCs and HKLs were removed from residual erythrocytes by incubating them 5 min with an osmotic shock sterile red blood cell lysis buffer (pH 7.4). The suspension was neutralized by PBS 1X (v: v) and centrifuged as indicated previously, then the leukocytes were collected and suspended in L-15 medium supplemented with 5% fetal bovine serum (FBS; Invitrogen), 1% Hepes (20 mM, Sigma, USA) and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A (PhA M form, Invitrogen).

Viable cells were adjusted to 5 x 10^6 cells mL^{-1} after enumeration using trypan blue stain (VWR, Leuven, Belgium) and seeded in wells of a 24 or 48-well plate (Greiner Bio-One, Vilvoorde, Belgium).

2.4. *In vitro* stimulation of cells and detection of humoral immune parameters

After isolation of striped catfish HKLs and PBMCs, five hundred µL of cell suspension ($5x10^6$ cells mL^{-1}) in L-15 medium supplemented with 5% FBS, 1% Hepes and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A were added to each well of 48-well plate (Greiner Bio-One, Vilvoorde, Belgium). Afterward, leukocytes stimulation was carried out with 20 ethanol plant extracts to reach final concentrations at 10 and 100 µg mL^{-1}. Cells cultivated in the same medium containing 0.5% DMSO served as control. Each experiment was realized in triplicates. The humoral immune response was assessed for 24 hps at 28°C in a humidified atmosphere of 5% CO_2. Collected leukocyte membranes were disrupted by 50 µL lysis buffer (50 mM tris HCl, 150 mM NaCl, 0.1% Triton X 100, PMSF 0.1 µg mL^{-1}). Samples were centrifuged at 2000 g for 10 min to remove debris. Supernatants were collected for immune assays.

2.4.1. Lysozyme assay

The lysozyme assay protocols were adapted from Ellis [49] and Milla *et al*. [50]. Briefly, 0.3 µg mL^{-1} *Micrococcus lysodeikticus* (Sigma) was suspended in phosphate buffer, pH 6.2. In 96 wells microplates, the lysozyme activity was measured after mixing 30 µl of cell culture supernatant with 120 µL of *M. lysodeikticus* suspension. The difference in absorbance at 450 nm was monitored in every 30 s, between 0 and 30 min. The absorbance measurements were used to calculate lysozyme activity in units. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

2.4.2. Complement assay

The alternative complement pathway (ACH50) was assayed using rabbit red blood cells (RRBC, Biomerieux, Craponne, France). The assay protocols were adapted from Sunyer and Tort [51] and Milla *et al*. [50]. Briefly, 3 µL of RRBC suspension (3%) diluted in veronal buffer (Biomerieux) were mixed with serial dilutions of cell culture supernatant (60 mL of total volume). After incubation for 120 min at 28°C, the samples were centrifuged at 2000 g for 10 min at room temperature. The spontaneous hemolysis was obtained by adding 60 mL of veronal buffer to 10 mL of RRBC. The total lysis was obtained by adding 60 mL of distilled water to RRBC. The absorbance of these samples was then measured at 405 nm. Appropriate calculations served to estimate complement activity.

2.4.3. Total immunoglobulin assay

The total immunoglobulin (Ig) concentrations of samples were measured by the method of Siwicki and Anderson [52], modified by Milla *et al.* [50]. Briefly, immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, Sigma). Serums were mixed with 12% PEG solution (v:v) for 2 h at room temperature under constant shaking. After centrifugation at 1000g for 10 min, the supernatant was collected and assayed for its protein concentration. The total immunoglobulin concentration was calculated by subtracting this value from the total protein concentration in the plasma before precipitation with PEG.

2.5. Detection of immunoregulatory genes production

Of the 20 herbal extracts tested, five strongly stimulated PBMCs and HKLs humoral immune responses were chosen for measuring immunoregulatory gene expressions. An *in vitro* study was performed to determine whether these five plant extracts can elicit different transcriptional profiles of the cytokines as well as antigen genes. Two mL of the PBMCs and HKLs suspension ($5x10^6$ cells mL^{-1}) in medium as mentioned above was added to each well of 12-well plate (Greiner Bio-One, Vilvoorde, Belgium), stimulated with five extracts as mentioned at 10 and 100 μg mL^{-1} and incubated for 6, 12, 24 and 48 h at 28°C in a humidified atmosphere of 5% CO_2. Cells collected containing 0.5% DMSO served as a control. Each experiment was performed in triplicates. Sampling of incubated cells was conducted at the time points mentioned above and the cells were stored at −80 °C prior to RNA extraction.

Table 2. Primers used for *P. hypophthalmus* immune gene expression

No.	Gene name		Primer Sequence (5'-3')	Calculated efficiencies (%)	Length (bp)	Accession number	Ref. Seq_Species
	Adaptive response						
1	a2 MHC class II integral membrane protein alpha chain 2		F: GAGCTCAACACTCAGCCAGT R: CACACCAGGAAGCTCCACAT	105	172	30783	*Danio rerio*
	Cytokine						
2	Interleukin-1β		F: CAGAGGCTGAAGCACACTCA R: CCTTGTCCTGCCTGCTGTAA	99	148	100304696	*Ictalurus punctatus*
3	Interferon γ	2a	F: TATGTCACTGAGCTGCTGGC R: TTAGCTTGACGTCGTCTCCG	96	143	N185453	*Pangasianodon hypophthalmus*
4		2b	F: TCCCAACCCTGCCAAATTGT R: GCCTCATTCTCCATCCAGGT	96	150	JN18545	
	Housekeeping Gene (Reference gene)						
5	16S rRNA		F: TATCTTCGGTTGGGGCG R: CCTGATCCAACATCGAGG	98	223	FJ432682	*Pangasianodon hypophthalmus*

For expression analysis, total RNAs were extracted from cells using Extract-All (Eurobio, Courtaboeuf, France) according to the manufacturer's protocols. Samples were then DNAse treated (DNA-free kit, Ambion, Austin, USA). The extracted RNA was quantified by spectrophotometry using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The RNA quality was assessed by the 260/280 and 260/230 ratios, while its integrity was evaluated by 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed using RevertAid HMinus First Strand cDNA synthesis Kit (Fermentas, Life Sciences, Germany). Briefly, 1μg of total RNA and random hexamer primers were denatured for 5min at 65 ◦C, then chilled on ice for 5min. Reverse transcription was performed in 20 μL of a final volume containing 4 μL of PCR buffer 5×, 20 units of ribonuclease inhibitors (1μL), 2μL of 10 mM dNTP Mix and 200 units of M-MuLV reverse transcriptase (1μL). The reaction was incubated for 60 min at 42 ◦C and stopped by heating at 70 ◦C for 5 min. The resulting cDNA was diluted 10x for initially testing the efficiency of primers combination or real-time quantitative PCR

Gene expression analysis

Three μL of reverse transcription products (diluted 1/10) were used for each real-time PCR. Duplicates were run for each sample. Forward and reverse primers were used at a concentration

of 600 nmol L^{-1} and added to SYBR Green PCR Master Mix (Applied Biosystem, Warrington, UK). The primers were designed for cytokine genes (*il1β*, *ifnγ* 2a and 2b) and adaptive immune cytokine (*mhc* class II) gene following previous paper of Sirimanapong *et al.* [21] and Sinha *et al.* [53] (table 2). After a 2 min incubation step at 50°C and a 10 min incubation step at 95◦C, 40 cycles of PCR were performed. The amplification parameters were as follows: 15 s of denaturing at 95◦C, 1 min annealing/extension at 60–64 °C depending on gene. The transcript abundance for each gene was calculated from the threshold cycle (CT) values using their respective standard curve followed by normalization with the geometric mean of ubiquitin and elongation factor. The expression was calculated according to the relative standard curve method of Plaffl [52], where $\Delta\Delta CT$ is $\Delta CT_{treatment} - \Delta CT_{control}$, ΔCT is $CT_{target\ gene} - C\ T_{16S,}$and CT is the cycle at which the threshold is crossed. Data are presented as relative fold-change with 16S rRNA as internal control genes

2.6. Statistical analysis

The statistical package for social science (SPSS) software (version 20.0, IBM Corp., Armonk, NY:IBM USA) was used to analyze differences between experimental and control groups. All data were expressed as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Tukey's test was run to find out any difference in immune parameters.

3. Results

3.1. Humoral immune parameters in PBMCs and HKLs

3.1.1. Lysozyme activity

Most examined extracts, which were selected by their previously known for influence on the immune response, stimulated the release of lysozyme activity after 24 hps in PBMCs and/or HKLs (Fig. 1). The lysozyme levels in PBMCs ranged between 0.85 and 4.79 (at 100 μg mL^{-1} of Ase and Mc, respectively) times than those of the control. Both doses of 10 and 100 μg mL^{-1} of Mc, Ob and Pg extracts significantly enhanced the lysozyme levels compared with control ($p < 0.01$) in PBMCs, while only those of Mc, Ob and Wc were effective on HKLs. The strongest effect was observed in PBMC treated with 100 μg mL^{-1} of Mc and in HKLs treated with 100 μg mL^{-1} dose of Ca ($p < 0.01$). Five extracts including Ar, Hc, Mp, Pb and Po showed no statistical influence on lysozyme activity in both PBMCs and HKLs.

3.1.2. Alternative complement pathway (ACH50)

As shown in Fig. 2, the ACH50 levels increased in cells treated with several extracts compared with control treatment at 24 hps (Fig. 2). At 100 μg mL^{-1}, PBMCs stimulated with Ai displayed the highest ACH50 value ($p < 0.01$), whereas Eh induced the strongest ACH50 activity in kidney cells at the same concentration, compared with other treatments. No significant changes were observed in both PBMCs and HKLs stimulated with Ac, Ca, Ep, Mc, Mp and Op, while both doses 10 and 100 μg mL^{-1} of Pa and Pg showed significant effects on PBMCs and those of Ase, Wc and Zo were effective on HKLs.

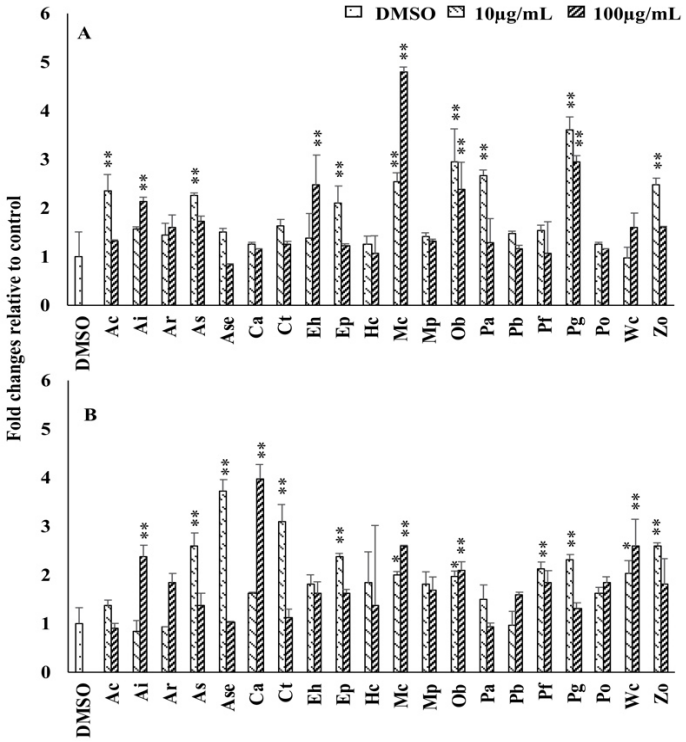

Figure 1. Lysozyme activity of A) PBMCs and B) HKLs of the striped catfish in the plant extract treated groups and control group (DMSO). Asterisk indicates significant differences in lysozyme levels between stimulated and unstimulated cells at 24hps (*$p < 0.05$; **$p < 0.01$). Values are mean ± S.D. (n=3).

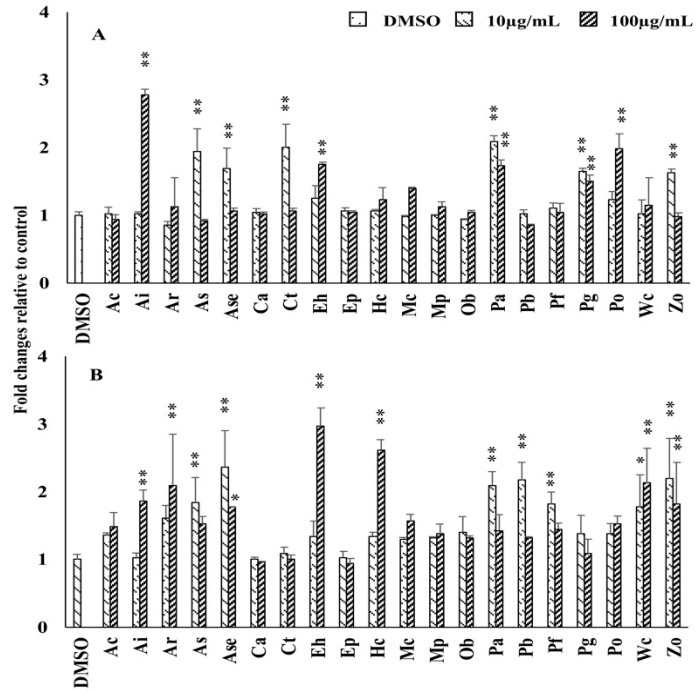

Figure 2. ACH50 of A) PBMCs and B) HKLs of the striped catfish in the plant extract treated groups and control group (DMSO). Asterisk indicates significant differences in ACH50 levels between stimulated and unstimulated cells at 24hps (*$p < 0.05$; **$p < 0.01$). Values are mean ± S.D. (n=3).

3.1.3. Total immunoglobulin (Ig)

In both PBMCs and HKLs, total Ig activity was noticed to be statistically higher in plant extract treated groups at 24 hps compared with control group (Fig.3) except Ca, Ep, Hc and Mc on PBMCs and Ar, Ca, Hc, Mp, Pg and Zo on HKLs. Most of the extracts could stimulate remarkably total Ig levels at single or both concentrations in PBMCs ($p < 0.01$). In case of HKLs, the level of total Ig was found to be the highest in Mc (at 100 µg mL^{-1}), Po (at 100 µg mL^{-1}), Pf (at 10 µg mL^{-1}), Ai (at 100 µg mL^{-1}) and Wc (at 100 µg mL^{-1}) ($p < 0.01$) treatments, while some seemed to decrease Ig activity (at one concentration Ca and Pa).

A compilation of the humoral immune results obtained in PBMCs and HKLs after stimulating with 20 plant extracts has been done (table 3) in order to select the 5 best extracts, namely As, Ai, Eh, Pa and Zo and analyzed more in depth the gene expression of 4 cytokines (*il1β*, *ifr*γ 2a and 2b) and a2 *mhc* class II integral membrane protein alpha chain 2.

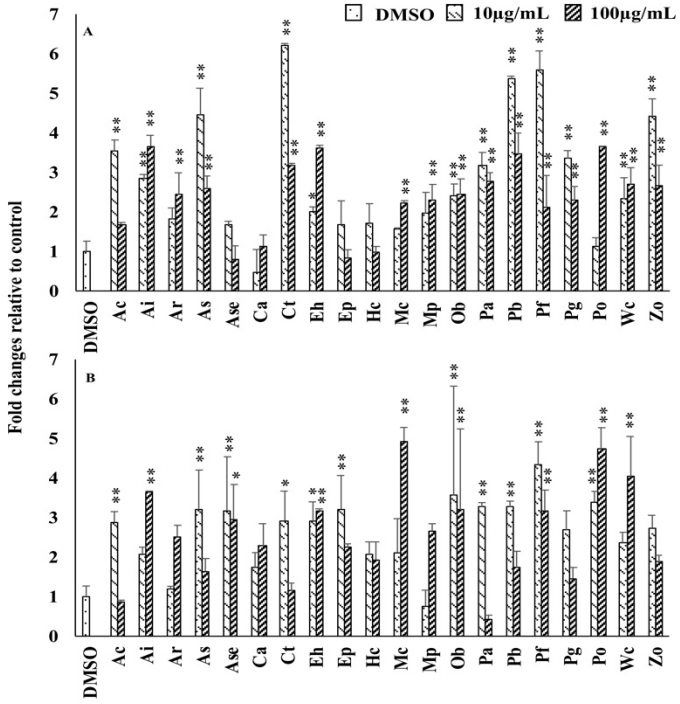

Figure 3. Total Ig of A) PBMCs and B) HKLs of the striped catfish in the plant extract treated groups and control group (DMSO). Asterisk indicates significant differences in Ig levels between stimulated and unstimulated cells at 24hps (*$p < 0.05$; **$p < 0.01$). Values are mean ± S.D. (n=3).

3.2. Gene expression

3.2.1. Expression of pro-inflammatory cytokine – *il1β*

The gene *il1β* was expressed in both cells types and its transcript levels were significantly enhanced by a stimulation with most plant extracts at least at some time points (Fig. 4). The levels of *il1β* reached a peak after 24-48 hps mainly in PBMCs or HKLs. In PBMCs treatments, the expression of *il1β* was the highest in Eh (100 µg mL^{-1}) treated groups at 48 hps (Fig.4A). When compared over time each individual treatment group, *il1β* levels displayed a wide

variation in response according to the plant species used. An early upregulation (at 6 hps) of *il1β* expression was observed only in Pa (10 μg mL^{-1}) treated group, gradually increasing until 24 hps ($p < 0.01$) and the highest expression level was observed in HKLs after 24 h in both doses of Pa stimulated. Furthermore, the *il1β* levels increased in single or couple doses of several extracts at some time points in HKLs, only Eh appeared no statistical increase compared with control group ($p > 0.05$) (Fig. 4B).

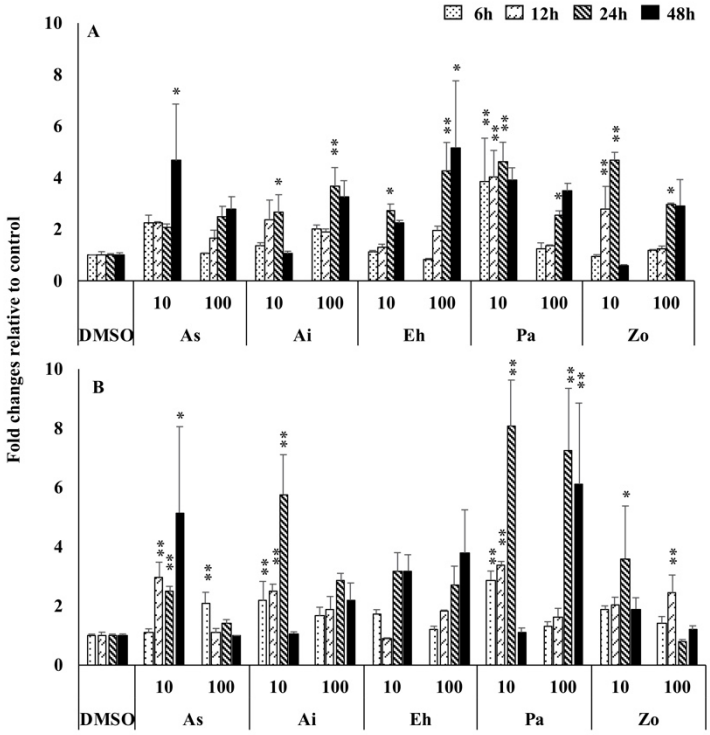

Figure 4. Expression (mean ± SD; n = 3) of *il1β* gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg mL−1). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$; **$p < 0.01$).

3.2.2. Expression of antiviral cytokines

In the present study, an upregulation of ifnγ 2a and 2b expressions was observed in PBMCs and HKLs for most extracts at one or several concentration/time point except Eh on PBMCs and Zo on HKLs. The expression of *ifnγ* 2a ($p < 0.01$) in PBMCs stimulated with Ai (100 μg mL^{-1}) increased remarkably from initial period till the end of the study and then stabilized (or decreased) until 48 hps. In case of cells treated with Pa at 10 μg mL^{-1}, the highest peak of *ifrγ* 2a level was observed at 24 hps ($p < 0.01$) (Fig. 5A). Similarly, Pa (10 μg mL^{-1}) also stimulated a significant increase of *ifnγ* 2a gene expression in HKLs at 12 hps ($p < 0.01$) (Fig. 5B).

Significantly higher expression level ($p < 0.01$) of *ifnγ* 2b was detected in PBMCs and HKLs incubated with the low dose of As or Pa while the activity at 100 μg mL^{-1} was reduced (Fig.6). High concentration of Ai also induced increased expression at some time points in both cell lines, while the effect of low dose was only significant after 24 hps on PBMCs at 10 μg mL^{-1}.

Eh also showed some increase of *ifnγ* 2b gene at 100 μg mL^{-1} after 48 hps on both cell lines, and Zo at the highest concentration on PBMCs after 24 hps.

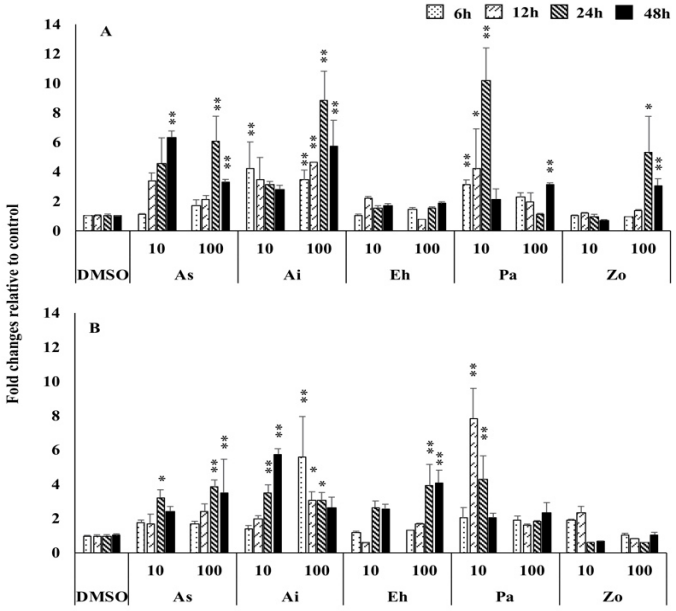

Figure 5. Expression (mean ± SD; n = 3) of *ifnγ* 2a gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg/mL). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$, **$p < 0.01$).

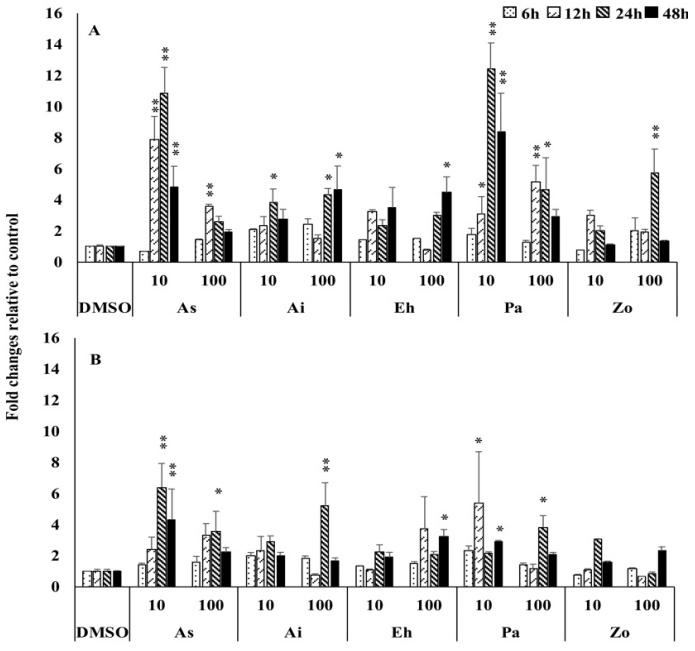

Figure 6. Expression (mean ± SD; n = 3) of *ifnγ* 2b gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg mL−1). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$, **$p < 0.01$).

3.2.3. Expression of cytokines involved in adaptive immunity-mhc class II

Inductive expression of *mhc* class II was obtained in PBMCs and HKLs treated with several extracts, from 6 hps onwards and often gradually elevated till 24 hps as compared with non-stimulated groups. Specifically, the *mhc* class II levels were upregulated early in stimulated PBMCs with As (10 μg mL^{-1}) and with both doses of Pa, then a prolonged and stable increase in their expression was observed until 24 hps, although there was a very slight decrease after 48 hps ($p < 0.05$) (Fig.7). *mhc* class II expression in HKLs was lower in comparison with the one of PBMCs, only treated cells with both doses of Pa showed an immediate rise in the expression level of *mhc* class II, and for Ai in some conditions. However, it was noticed that three extracts (As, Eh and Zo) failed to stimulate the expression of *mhc* class II in HKLs, regardless of the dose used.

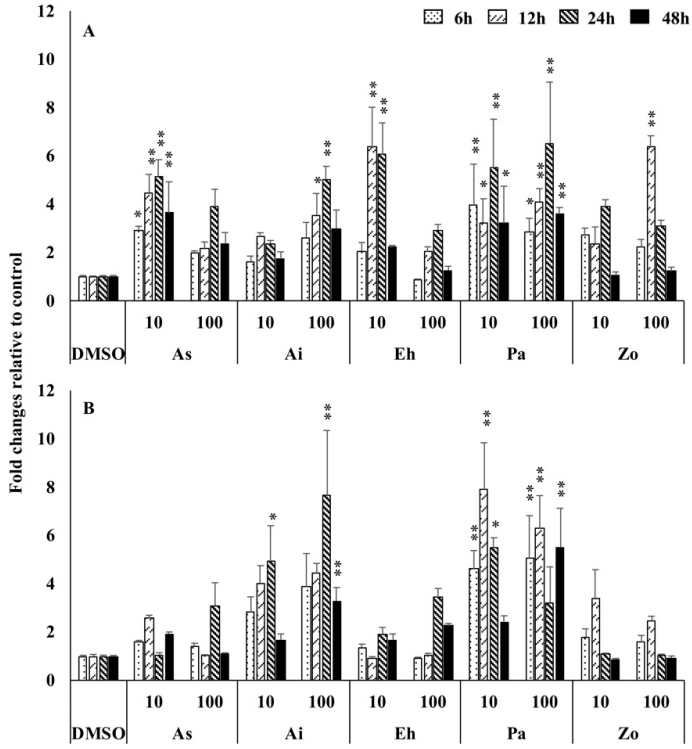

Figure 7. Expression (mean ± SD; n = 3) of *mhc* class II gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg mL−1). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$, **$p < 0.01$).

4. Discussion

Plant products are a great source of bioactive molecules and some have been identified as potential therapeutic treatments by modulating immunity as well as by preventing or controlling fish diseases [26, 29, 55]. In the past few years, aquaculture of striped catfish has been suffering many troubles due to the disease threats, especially, infections, caused mostly by bacteria (e.g. *A. hydrophila* and *E. ictaluri*) [56-59]. However, research about the effects of plant extracts on immune molecules as well as on humoral endpoints in striped catfish is rather limited. The present study showed the results of screening the putative immune-stimulatory

properties of 20 plant extracts on the PBMCs and the HKLs, which are well known for their involvement in innate and acquired immunities. Functional evaluation through *in vitro* studies were performed by measuring the lysozyme, complement activities and total Ig as well as the cytokine gene expression with colorimetric assays and molecular tools. This research further determined the mode of actions of some ethanol plant extracts in striped catfish leukocytes by expression of some cytokines at different time points.

Table 3. Summary of the humoral immune responses in PBMCs and HKLs stimulated with the 20 selected plant extracts.

No.	Name	Dose (µg/ml)	Lysozyme		Complement		Total Ig	
			PBMCs	HKLs	PBMCs	HKLs	PBMCs	HKLs
1	Ac	10	**				**	**
		100						
2	Ai	10					**	
		100	**	**	**	**	**	**
3	Ar	10						
		100				**	**	
4	As	10	**	**	**	**	**	**
		100					**	
5	Ase	10		**	**	**		**
		100				*		*
6	Ca	10						
		100		**				
7	Ct	10		**	**		**	*
		100					**	
8	Eh	10					*	
		100	**		**	**	**	**
9	Ep	10	**	**				**
		100						
10	Hc	10						
		100				**		
11	Mc	10	**	*				
		100	**	**			**	**
12	Mp	10						
		100					**	
13	Ob	10	**	*			**	**
		100	**	**			**	**
14	Pa	10	**		**	**	**	**
		100			**		**	
15	Pb	10				**	**	**
		100					**	
16	Pf	10		**		**	**	**
		100					**	**
17	Pg	10	**	**	**		**	
		100	**		**		**	
18	Po	10						**
		100			**		**	**
19	Wc	10		*		*	**	
		100		**		**	**	**
20	Zo	10	**	**	**	**	**	
		100				**	**	

Data are presented as means ± SD. (*) Asterisks indicate significant increased between the experimental groups to control, when $p < 0.05$ (*), $p < 0.01$ (**). Yellow shading includes the extracts for which at least 5 immune responses were significantly increased compared to control at 24hps. PBMCs: peripheral blood mononuclear cells; HKLs: head kidney leukocytes

Playing a role as indicators of inflammatory response, humoral immune parameters (lysozyme, complement and total Ig) were measured in two types of striped catfish leukocytes stimulated with plant extracts at 24 hps. Among these parameters, lysozyme is important in host mediating protection against microbial invasion, and is mainly expressed in neutrophils, monocytes and in small amount in macrophages [60]. In the present study, Ai, As, Eh, Mc, Ob, Pg and Zo stimulated to increase significantly lysozyme levels in both striped catfish leukocytes at single or couple concentrations at 24h. A similar result was documented by Giri *et al.* [61] who reported that significant levels of lysozyme started to display in roho labeo head kidney macrophages at 24h post stimulating with intracellular products of the probiotics *Bacillus subtilis* VSG1 and *Lactobacillus plantarum* VSG3. Lysozyme activities are immune effectors that could eliminate the widespread bacteria and viruses [62, 63]. There are two major c-type and g-type lysozymes caused degree of lysozyme activity, which could be induced by LPS, bacterial, fungal and some viral infection [64, 65]. In HKLs of Japanese pufferfish (*Takifugu rubripes*) stimulation by LPS (20 μg mL^{-1}) and Nigericin (30 μM), however, the lysozyme activity was observed to increase at 24 h, later than phagocytosis and superoxide anion production, then the levels continued to increase until 48 hps [66]. Similar to lysozyme parameter, our study also demonstrated that Ai, As, Ase, Eh, Pa and Zo extracts remarkably induce complement activity in both striped catfish PBMCs and HKLs. Aside with mediating humoral immunity, fish complement and immunoglobulin can have a synergistic effect on the microbial opsonization [67-69]. IgM is the common immunoglobulin in both serum and mucus which plays a key role in systemic immune responses [70]. Klesius [71] and Magnadóttir *et al.* [72] indicated that the IgM levels are variable among fish and with size/age. For this reason, total Ig is used as an immune parameter evaluated for medical immunostimulants. Previous studies suggested that total Ig levels were well regulated in fish by dietary extracts including Astragalus (*Astragalus membranaceus)* and wolfberry (*Lycium barbarum*) [73], rose myrtle [74-76]. Similar result was observed in which our study, several plant extracts could statistically regulate the total Ig level in both striped catfish cell types, mostly after 24 hps. These results indicated that the modulation of humoral immune responses (lysozyme and complement activities, total Ig) was activated in striped catfish by plant extracts but might differ between the kinds of plant extracts and leukocyte types (PBMCs and HKLs).

Nevertheless, the understanding of the molecular mechanisms behind the effects of plant extracts on fish immune response is undoubtedly helpful for proposing appropriate applications in fish farming. In this study, 5 plant products (As, Ai, Eh, Pa and Zo) were selected from the lysozyme, complement and total Ig results to determine the mRNA levels of immune-related genes in PBMCs and HKLs (Table 3). This study showed that most of the immune cytokine genes were upregulated in PBMCs and HKLs stimulated with these extracts, but this stimulation often depends on the concentration, with the highest concentration sometimes less effective than the lowest one. Effects were also sometimes lower after 48 hps. Like in mammals, teleost IL-1β has been found to regulate other immune relevant genes in lymphocyte activation, phagocytosis and bactericidal activities after stimulation with its recombinant protein [77-80]. Macrophages are the primary source of IL-1β although it is produced by a

wide variety of other cell types as well [81]. The incubation of trout anterior kidney leukocytes with recombinant Il-1β induces phagocytosis and chemotaxis [79, 82]. In the present study, *il1β* expression levels in striped catfish leukocytes varied over the experimental period according to the type of extract treatment. Although the *il1β* gene started to significantly increase at the initial hours after stimulation by some extracts when compared with control, the highest peaks of *il1β* expression were observed in most plant extract-treated cells after 24 or 48 hps. Similarly, upregulated expression of *il1β* was noticed at the very early stage in Japanese pufferfish HKLs after incubation with heat-killed probiotics [44]. A study by Bilen *et al.* [66] also found that the expression of pro-inflammatory cytokine genes in Japanese pufferfish HKLs increased after treated with nigericin, a combination of nigericin and LPS. However, these authors demonstrated that the *il1β* levels of Japanese pufferfish HKLs significantly increased very early at 1 hps, and the upregulation was sustained until 24 hps. In addition, rainbow trout head kidney macrophages treated with rose myrtle led to the upregulation of *il1β* after 4 hps only [43]. Moreover, Chi *et al.* [83] also confirmed an upregulation of *il1β* and *tnfα* mRNA levels in HK macrophages of grass carp (*Ctenopharyngodon idella*) after a stimulation with wood ferm *Dryopteris crassirhizoma* extract at 2 and 8 hps. This could be explained that the regulation of *il1β* level was varied in cells according to stimulants and kind of cell types.

As a crucial cytokine in immune mechanism, IFNs also provides mediating cellular defense against viral infections. Especially, the type II IFN (IFN-γ) plays a major role in both innate and adaptive immunity, including the ability to activate respiratory burst activity, nitric oxide production and phagocytosis *o*f bacteria in macrophages [84-86]. Channel catfish (*Ictalurus punctatus) ifn* expression increased at 2 h after exposure to an inactivated double-stranded RNA retrovirus and poly-inosinic : cytidilic acid (poly I:C) [87]. In correlation with the increased *il1β*, the current results found that *ifn*γ 2a and 2b expressions were upregulated by extract treatments mostly at 12 hps. Biswas *et al*. [44] found that type I- *ifn1* gene expression in Japanese pufferfish HKLs was induced by both heat-killed probiotics (*Lactobacillus paracasei* spp. *paracasei* - strain 06TCa22 and *L. plantarum* - strain 06CC2), whereas *ifn*γ gene expression was increased by *L. paracasei* spp. *paracasei* at different time points (4, 8, 12, 24 and 48 hps). Furthermore, upregulation of *ifn*γ2 gene was also induced by yeast extract administration to common carp, whereas induction of type I *ifn* (*ifn*α) expression was not detected [88]. The increased *ifn*γ levels observed in this study demonstrated that extract treatments could protect fish against viral infection and give striped catfish leukocytes the ability of killing intracellular pathogens by *ifn*γ mediation. On the other hand, expression of *mhc* class II gene was also obtained in PBMCs and HKLs treated with several extracts, although HKLs were less stimulated in comparison with PBMCs. Class II MHC plays a vital role by mediating antigen recognition in macrophages, B-cells and dendritic cells [89]. The *mhc* genes have been isolated and characterized in various fish species, including zebrafish (*Danio rerio*), rainbow trout, channel catfish, turbot *(Scophthalmus maximus)*, Nile tilapia (*Oreochromis niloticus*), sea bass (*Centropristis striata*) and half smooth tongue sole (*Cynoglossus semilaevis*) [90] and European eel (*Anguilla anguilla*) [91]. Jiang *et al.* [92] found that channel catfish head kidney monocytes/macrophages were stimulated by three

flagellins (rFlaA, rFlaB and rFlaC). These flagellins could dramatically upregulate *mhc* class *II* level at 8 hps. The *mhc* class II genes were highly expressed compared with the other examined genes (namely toll-like receptor 5- *tlr5m, tlr5s, ηfκB, il1β, tnfα, il8, inos1*). Furthermore, class II *mhc* levels were strongly induced in LPS stimulated-macrophage of half smooth tongue sole at 12 hps [93].

Plant extracts possess many medicinal functions that could be due to alkaloids, steroids, phenolics, tannins, terpenoids, saponins, and flavonoids presented in the extracts [23-30]. A study by Catap et al, [94] indicated that the presence of tannins and alkaloids in the extract could produce the immunomodulatory effects in lymphocytes and macrophages. Likewise, alkaloids induced the proliferation of lymphocytes, but not at a significant level [94]. Furthermore, an *in vitro* study using Leishmania-infected RAW 264.7 cells suggested that tannins played as an indicator in activating macrophages, especially during infections [95]. Tannins have been reported to enhance innate immunity through proliferation of γδ T lymphocytes as a result of providing host protection against pathogens [96]. These could be explained why striped catfish leukocytes can improve the immune systems after being stimulated with plant extracts.

In conclusion, our results suggest a positive contribution of several herbal extracts to increase humoral immune responses in a dose dependent manner in striped catfish PBMCs and HKLs after 24 h. Nevertheless, the humoral immune responses cannot adequately explain the stimulatory effects of these extracts. Several extracts induced a strong upregulation of 4 cytokines (*il1β*, *ifnγ* 2a and 2b, and *a2 mhc* class II) according to the concentration, time points and kind of leukocytes. The specific enhancement of the gene expression by the plant extracts might promote the activity of fish macrophages leading to an early protective immunity during disease infection. Furthermore, this study demonstrates the ability of these plant extracts to enhance the activation of immune response and suggests a new mechanism explaining their important biological characteristics of plant extracts (*e.g*. antibacterial, antiviral, anti-inflammatory or immune-stimulating properties in fish).

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Chapter 5

Plant extract-based diets differently modulate immune responses and resistance to bacterial infection in striped catfish (*Pangasianodon hypophthalmus*)

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Hypothesis outlines

We previously evaluated the effects of 20 plant extracts on immune responses of striped catfish leukocytes (Chapter 4). These extracts were also examined for their functions in antioxidant activity, which was done by PhD student Nguyen Le Anh Dao. After the first comparison of *in vitro* tests, we found that extracts including *Euphorbia hirta* and *Phyllanthus amarus*, and *Psidium Guajava* could strongly stimulate both immune responses and antioxidant functions. *Mimosa pudica* extract was less effective to immune responses while the extract was good in antioxidant activity. In contrast, *Azadiracha indica* extract enhanced better the immune parameters in striped catfish leukocytes, whereas it could not improve the antioxidant activity. Moreover, the five extracts were easy to be collected in Mekong Delta of Viet nam with the huge quantity for the in vivo experiment. These five extracts at two different doses were continuously applied to striped catfish as oral administration of extract-based diets for 8 weeks. The doses of extracts were selected in combination between bibliographic review and *in vitro* screening test. Based on the *in vitro* screening study, three extracts including *M. pudica*, *E. hirta* and *A. indica* were mostly the best immune response in leukocytes at the high concentration (100 µg/mL), whereas *P. guajava* and *P. amarus* extracts could stimulate immune response in those cells at the low concentration (10 µg/ml). Moreover, several publications reported the concentrations of *P. guajava* usually ranged from 0.1 to 1% when supplemented in fish diets and *E. hirta* concentrations ranging from 0.5 to 5%. Taken together, we decided to perform *in vivo* experiment with striped catfish at 0.2 and 1.0% of *P. guajava* and *P. amarus*; and 0.4 and 2.0 % of *M. pudica*, *A. indica* and *E. hirta*. During the *in vivo* experiment, the effects of some selected plant extracts will be determined under laboratory conditions and the beneficial effects will be evaluated in terms the immunological biomarkers (lysozyme, complement, and total immunoglobulin), and hematological biomarkers, as well as bacterial resistant capacity. The effects of five plant extracts in the *in vivo* experiment will be validated and compared with *in vitro* tests to suggest the best plant. The question of the consistent effects between *in vitro* and *in vivo* tests will also be answered.

Abstract

A feeding trial was performed to compare the effects of five ethanol herbal extracts (bhumi amla, *Phyllanthus amarus* Schum and Thonn [Pa]; guava, *Psidium guajava* L. [Pg]; sensitive plant, *Mimosa pudica* L. [Mp]; neem, *Azadirachta indica* A. Juss [Ai] and asthma plant, *Euphorbia hirta* L. [Eh]) on the immune response and disease resistance against *Edwardsiella ictaluri* infection of striped catfish (*Pangasianodon hypophthalmus*). Fish were fed diets supplemented with two doses of each plant extract (0% [basal diet], 0.4% Eh [Eh0.4], 2.0% Eh [Eh2.0], 0.2% Pa [Pa0.2], 1.0% Pa [Pa1.0], 0.2% Pg [Pg0.2], 1.0% Pg [Pg1.0], 0.4% Mp [Mp0.4], 2.0% Mp [Mp2.0], 0.4% Ai [Ai0.4], 2.0% Ai [Ai2.0]) for 8 weeks. Results showed that hematological parameters (total red blood cells, white blood cells, lymphocytes, monocytes, and neutrophils) of fish fed extract-based diets were significantly higher than in those fed the control diet ($p < 0.05$) after 4 and 8 weeks. Plasma lysozyme activity increased in fish whose diets contained both doses of Eh ($p < 0.05$) in week 4 (W4), whereas lysozyme activity increased in fish fed 0.2% Pa and Pg, and 2.0% Ai and Eh ($p < 0.05$) in week 8 (W8). The lysozyme levels in skin mucus did not significantly differ between treatments ($p > 0.05$) in W4 and after the bacterial challenge test. At the end of the feeding trial, levels of ACH50 significantly increased in most of extract groups compared to the control group ($p < 0.05$). Total immunoglobulin increased considerably in both the plasma and skin mucus of fish fed extract-supplemented diets after 8 weeks. In addition, dietary supplementation with Pg, Mp, Pa0.2, Eh2.0, and Ai0.4 for 8 weeks considerably reduced the cumulative mortality against *E. ictaluri* infection in striped catfish. The results suggest that plant extracts possibly modulate the striped catfish immune response in a time and dose dependent manner. Specifically, diets enriched with extracts of *P. guajava* at 0.2 and 1.0%, or *M. pudica* at 2.0% for 8 weeks, have great potential for improving striped catfish health by enhancing the immune system and reducing mortality against bacterial challenges.

1. Introduction

The striped catfish (*P. hypophthalmus*) is a migratory riverine species that has become an economically important fish and is widely cultured in several Asian countries. Striped catfish aquaculture has been developing rapidly in Vietnam, with an annual production that exceeded 300 thousand tons in 2004 to more than 1.2 million tons in 2018 [1, 2]. However, intensive striped catfish production induces environmental stressors due to high stocking densities, and consequently increases mortality rates due to bacterial infections. Recent studies have reported that *E. ictaluri* is one of the most common bacterial pathogens of striped catfish and is responsible for huge economic losses at fingerling stages [3, 4]. Understanding how the specific and non-specific immune responses modulate fish health is a key to improve productivity and reduce losses in the intensive aquaculture sector. Several immunostimulants used as diet additives could enhance striped catfish defense mechanisms and thus prevent losses from diseases [5-8]. Moreover, boosting the immune response with ecologically friendly compounds is an effective strategy to promote sustainable aquaculture.

From this perspective, bio-products resulting from natural plants have attracted considerable

attention as a source of eco-friendly prophylactic compounds for using in the aquaculture industry. To date, many medicinal plant products have been demonstrated to possess a wide range of active components such as alkaloids, steroids, phenols, tannins, terpenoids, saponins, glycosides, and flavonoids [9, 10], although their ingredients have not yet been completely described [11]. These compounds are responsible for potential bio-activities in fish [12], including growth promotion, appetite stimulation, immune stimulation, antimicrobial, and anti-stress effects [13-15]. Previous research reports that plant extracts have been successfully applied to improve aquatic organisms' immunity and disease resistance [16-19]. In fish, three different routes of administration have been tested, including injection, immersion, and oral, and the latter tends to be more practical than the others [20-22].

Innate and adaptive immune responses are considered to be the essential functions of fish defense mechanisms. The epithelial/mucosal barrier, and humoral and cellular immunity are the three main components of fish immunity [23]. The innate humoral parameters are represented by lysozyme, hemolysin, and complement molecules, etc., which are commonly high in fish plasma [24-26] ; the key humoral parameter of the adaptive system is the immunoglobulins, expressed mainly as B-lymphocyte receptors or secreted in plasma [23]. Additionally, the mucosal layer of the fish skin also functions as the primary barrier between a fish and its external environment, and thus plays an important role in fish health protection. Physical properties of the skin mucus (including lysozyme, immunoglobulins, complement, agglutinins, and lysins) act as biologically active molecules in fish [27, 28]. Earlier studies have reported that dietary supplemented with ginger (*Zingiber officinale* Roscoe) [29], garlic (*Allium sativum)* [30] and extracts of date palm fruit (*Phoenix dactylifera* L.) [31] promoted skin mucosal immune responses in rohu (*Labeo rohita*), Caspian roach (*Rutilus rutilus*), and common carp (*Cyprinus carpio*), respectively. The mode of action of these herbs is usually the enhancement of the immune response through the elevation of immune parameters, and control of infectious diseases by mitigation of many side effects involving the synthesis of antimicrobials [32]. Vietnam has plenty of wild plant resources distributed in the different eco-regions [33]. However, the use of natural products in aquaculture is not yet popular in the country. Farmers lack knowledge regarding the existence of such bio-active products or their efficacy in fish. This study was conducted to investigate whether five ethanolic herbal extracts (*E. hirta, P. amarus*, *P. guajava*, *M. pudica* and *A. indica*) affect the modulation of innate and adaptive immune responses and disease resistance against *E. ictaluri* infection in striped catfish.

2. Material and methods

2.1. Extract preparation

Fresh parts of *E. hirta* [Eh] (leaves, twigs), *P. amarus* [Pa] (leaves, twigs), *M. pudica* [Mp] (leaves, twigs), *P. guajava* [Pg] (leaves), and *A. indica* [Ai] (leaves) were collected from the Mekong Delta in Vietnam. The plants were authenticated at the Department of Biology, College of Natural Science, Can Tho University. All collected plant parts were washed to remove mud and dust, and rotten and damaged parts were discarded. Plants were air dried in

shade for several days and then in an oven at about 60°C until well-dried. Plants were then ground to fine powders in a blender and stored in sealed containers in a cool, dry place.

The dried powder (100 g) was soaked in ethanol 96% (800 mL) for at least 24 h at room temperature with frequent agitation. The solvent-containing extracts were then decanted and filtered. The ground samples were further extracted 4 times with ethanol 96%. The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator to give crude ethanol extracts. All the well-dried crude ethanol extracts were stored at -20°C until use.

2.2. Diet preparation

The basal diet was prepared according to Table 1. The diet contained 30% crude protein, 6.66% crude lipid, 10.58% ash, 3.21% fibre, and 4.41 kcal/g energy. Fishmeal, soybean meal, cassava, and rice bran were mixed and sterilized at 110°C for 10 min (Mixture A). Butylated hydroxytoluene (BHT), vitamins and minerals were well mixed with each of the plant extract concentrations (Mixture B). Then mixture A was mixed with mixture B and fish oil. The final mixture was extruded through a mini-extrusion machine (Can Tho University, Vietnam) at 70°C without steaming. Basal diets were supplemented with the different plant extracts at two concentrations for each plant extract as described above. The experimental feeds were then dried at 60°C for 24 h to achieve 11-12% moisture. Pellets of 2 mm were stored at –20°C until use.

2.3. Bacteria preparation

E. ictaluri strain- Ed1 isolated from striped catfish in Mekong Delta of Vietnam were cultured on tryptic soy agar plates (Merck, MA, USA) for 48 h at 28°C following the method of Hang et al. [5]. Then, a single colony was collected and harvested into tryptic soy broth (Merck, MA, USA). This suspension was shaken overnight, 180 rpm at 28°C. Then, bacteria were centrifuged at 5000 rpm at 4°C for 5 min and washed 3 times with 0.85% NaCl solution. The mean colony count was found using the optical density method [34] by spectrophotometer (Thermo spectronic, USA) at 590 nm, and OD value was adjusted to 0.1. This suspension was diluted 1000 times with NaCl solution and injected to the fish.

2.4. Fish acclimatization and feeding trial

Farm-raised striped catfish juveniles (15-20 g) were obtained from a local fish farm in Vinh Long province, Vietnam, and transported to the laboratory in plastic bags filled with oxygenated water. The fish were acclimatized to laboratory conditions for 15 days then maintained into composite tanks (250 L) under a flow-through freshwater supply system, and fed twice a day with the formulated diets at a rate of 2% of their body weight/day.

For the feeding trial of plant extract-based diets, fish were randomly divided into 11 treatments, with each treatment given in triplicate. Fish were fed the experimental diets for 8 weeks, at 2% of body weight and three times (8am, 12am, and 5pm) daily. Tank capacity was 250 L, and each tank contained 50 fish. The photoperiod was of 12 h light: 12 h dark. Water temperature,

dissolved oxygen, and pH were monitored daily and maintained throughout the experimental period at 30 ± 2°C, 5.7 ± 0.01 mg /L, and 7.5 ± 0.02 respectively.

After 8 weeks of feeding, all groups (45 fish per group) fed plant extract-based diets were injected intraperitoneally (i.p.) with 0.1 mL LD50 of *E. ictaluri* suspension. At the same time, the control groups were divided into two small groups, the first group was the control injected with 0.1 mL of 0.85% NaCl solution and the second group was challenged with 0.1 mL LD50 of *E. ictaluri*. All groups were maintained in triplicate, 15 fish per tank. Cumulative mortality was recorded daily for 14 days after the challenge test. To ensure that mortalities were due to bacterial infection, *E. ictaluri* was re-isolated and identified by PCR confirmation.

2.5. Sample collection

The skin mucus samples were collected at W4 and W8 of the feeding trial, and 3 days post injection (dpi) according to the method by Ross et al. [35] with slight modification. Briefly, 3 fish per tank were randomly collected and anaesthetised using 0.1 ppm M222 (Sigma–Aldrich, MO, USA). Fish surfaces were individually washed with distilled water and then transferred into polyethylene bags containing 1 mL of PBS 1X. After 2 min of gentle shaking, mucus was collected, transferred to 2.0 mL sterile eppendorf tubes and centrifuged (1500 × *g* for 10 min at 4°C). The supernatant was stored at –80°C for further analysis. Similarly, blood samples were obtained from the caudal vein of individual fish (9 fish per treatment, 3 fish per tank) and centrifuged at 4000 rpm for 10 min. The plasma supernatant was collected into new eppendorf tubes and kept at –80°C until analysis.

2.6. Hematology

Total red blood cell (RBC) counts were made with a Neubauer hemocytometer using Natt-Herrick solution as a diluent stain [36]. First, 10 µL of each blood sample was diluted in 1990 µL of Natt and Herrick's solution and mixed gently for at least 3 min. The cell suspension was put into the chamber and allowed to settle for 2–3 min before initiating a count under the microscope light. The RBCs were counted in 5 out of the 25 small areas. White blood cell (WBC) types were identified by smearing a small drop of whole blood on a microscope smearing slide (cover glasses 22 × 22 mm, Germany). The slide smear was quickly dried, fixed in methanol (95%, Merck, MA, USA) for 1–2 min and stained with Wright's or Giemsa (Merck, MA, USA) [37]. Classification of blood cell types was determined following Supranee et al. [38]. Results for each blood cell were calculated according to Hrubec et al. [39].

2.7. Lysozyme assay

The lysozyme assay protocol was adapted from Ellis [40] and Milla et al. [41]. In 96-well microplates, the lysozyme activity assay was initiated by mixing 10 µL of plasma or 20 µL of skin mucus with 130 µL of lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich, MO, USA) suspension in phosphate buffer, pH 6.2 (0.6 mg/mL for plasma and 0.3 mg/mL for skin mucus). The difference in absorbance at 450 nm was monitored between 0 and 30 min for plasma (0 and 15 min for the skin mucus) and used to calculate units of lysozyme activity. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

Table 1. Composition of experimental diets

Ingredients (100 g of feed)		**Experimental diets**									
	Control diet	**Pa0.2**	**Pa1.0**	**Pg0.2**	**Pg1.0**	**Ai0.4**	**Ai2.0**	**Mp0.4**	**Mp2.0**	**Eh0.4**	**Eh2.0**
[a]Soybean meal (g)	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62
[b]Rice bran (g)	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5
[c]Casava (g)	18.36	18.16	17.36	18.16	17.36	17.96	16.36	17.96	16.36	17.96	16.36
[d]Fishmeal (g)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
[e]Fish oil (g)	1	1	1	1	1	1	1	1	1	1	1
[f]Premix* (g)	3	3	3	3	3	3	3	3	3	3	3
[g]Gelatin (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
[h]Butylated hydroxytoluene (BHT)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Plant extracts (g)											
Pa	–	0.2	1.0	–	–	–	–	–	–	–	–
Pg	–	–	–	0.2	1.0	–	–	–	–	–	–
Ai	–	–	–	–	–	0.4	2.0	–	–	–	–
Mp	–	–	–	–	–	–	–	0.4	2.0	–	–
Eh	–	–	–	–	–	–	–	–	–	0.4	2.0

Pa: *Phyllanthus amarus*, Pg: *Psidium guajava*, Mp: *Mimosa pudica*, Ai: *Azadirachta indica*, Eh: *Euphorbia hirta*

[a]Wilpromil R Soy Protein Concentrate, Yihai (Fangchenggang) Soybeans Industries, (Wilmar Group), Fangchenggang, China.

[b]Cai Lan Oils & Fats Industries Company, Can Tho Branch, Can Tho City, Vietnam.

[c]Hong Ha Company, Can Tho City, Vietnam.

[d]Minh Tam, Can Tho, Vietnam.

[e]Vegetable oil (Simply, Vietnam) and squid oil (Vemedim, Vietnam) at a ratio of 1:1.

[f]The vitamin/mineral premix (Unit/kg) from Vemedim, Can Tho, VietNam: vitamin A, 6000 IU; vitamin D3, 5600 IU; vitamin E, 160 IU; vitamin B1, 10 mg; vitamin B6, 20 mg; vitamin B12, 0.03 mg; vitamin K, 0.3 mg; riboflavin, 60 mg; vitamin C, 300 mg; pantothenic acid, 60 mg; folic acid, 8 mg; nicotinic acid, 184 mg; biotin, 0.3 mg; iron, 50 mg; copper, 10 mg; iodine, 9 mg; zinc, 34 mg; selenium, 0.4 mg; manganese, 30 mg

[g]Xilong Chemical Industry Incorporated (China)

[h] Honshu Chemical Industry Company, Japan

2.8. Complement assay

The plasma alternative complement pathway was assayed using rabbit red blood cells (RRBC, BioMérieux, France) as targets following Sunyer and Tort [42] and adapted by Milla et al. [41]. Briefly, 10 µL of RRBC suspension (3%) diluted in veronal buffer (BioMérieux, Marcy l'Étoile, France) were mixed with serial dilutions of plasma (60 µL total volume). After incubation for 100 min at 28°C, the samples were centrifuged at 2000 × *g* for 10 min at room temperature. Spontaneous hemolysis was obtained by adding 60 µL of veronal buffer to 10 µL of RRBC. Total lysis was obtained by adding 60 µL of distilled water to RRBC. The absorbance was measured at 405 nm. Appropriate calculations served to estimate complement activity.

2.9. Total Ig assay

The total immunoglobulin concentration of each sample was measured using the method of Siwicki and Anderson [43], modified by Milla et al [41]. Briefly, immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, Sigma–Aldrich, MO, USA). Plasma or skin mucus was mixed with 12% PEG solution (v:v) for 2 h at room temperature under constant shaking. After centrifugation at 1000 × *g* for 10 min, the supernatant was collected and assayed for its protein concentration. The total immunoglobulin concentration was calculated by subtracting this value from the total protein concentration in the plasma or mucus before precipitation with PEG.

2.10. Bacteria detection

DNA extraction

Head kidneys of fish were mixed and ground with 600 µL lysis buffer (0.5 M NaCl, 0.1 M Tris–HCl at pH 8.0, 1% sodium dodecyl sulfate, and 0.1 mM EDTA) and 2.5 µL of proteinase-K solution (40 mg/mL). All mixtures were mixed well and incubated for 15 min at 37°C and then 2.5 µL of RNase (2 mg/mL) was added, mixed and incubated for 30 min at 37°C. Upon addition of 600 µL chloroform: isoamylalcohol (24:1, v:v), the mixtures were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was collected in a new tube. Following addition of 600 µL phenol: chloroform: isoamylalcohol (25:24:1), the mixtures were mixed well and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and quickly mixed with 500 µL cold isopropanol, and centrifuged at 13,000 rpm for 10 min. The DNA pellets were washed once with 70% ethanol and dried. Before PCR, DNA was dissolved in TE buffer (10 mM Tris–HCl and 0.1 mM EDTA at pH 8.0) and stored at –20°C.

PCR amplification

A PCR reaction was performed to amplify a 407 bp specific DNA fragment of *E. ictaluri* with forward primer 5′-GTA GCA GGG AGA AAG CTT GC-3′ and reverse primer 5′-GAA CGC TAT TAA CGC TCA CAC C-3′ [44]. Each 25 µL reaction contained 1.5 mM $MgCl_2$, 0.2 nM dNTPs, 0.4 µM of each primer, 2.5 U of Taq polymerase (Promega, Madison, USA), and 100 ng of DNA extracted from fish head kidneys. PCR amplification was performed using a thermocycler (Applied Biosystems). The cycling parameters consisted of an initial

denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 45 s and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. PCR amplicons were resolved by agarose gel electrophoresis in 1% agarose in 40 mM Tris–acetate, 1 mM EDTA, and stained with 1 mg/mL ethidium bromide.

2.11. Statistical analyses

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY:IBM USA). The normality of the data and the homogeneity of variance between groups were tested using Shapiro-Wilks and Levene tests. Results are presented as means ± SEM (standard error of the means). One-way ANOVA analysis of variance Duncan's multiple range test at a confidence level of 95% ($p < 0.05$) was used to determine significant differences between immunological variables in fish from the different plant extract treatments and control treatment.

3. Results

3.1. Effect of extract-supplemented diets on blood parameters of striped catfish

The hematological parameters significantly increased ($p < 0.05$) in a dose-dependent manner in all extract treatments compared to those of the control (Fig. 1). The number of RBCs did not show any significant differences between groups at W4, but the value was statistically higher than control group in the Pg1.0 group in W8. The RBC abundance tended to decrease in all experimental groups after *E. ictaluri* infection. Pa0.2 group significantly reduced the number of RBCs, whereas the RBC counts was statistically higher in the Mp2.0 group compared to control treatment ($p < 0.05$) (Fig. 1A).

For all sampling times, the WBC numbers statistically increased in Pg groups and in the Mp2.0 group compared to control. Extract-based diets with Pa and Ai did not enhance the number of WBCs in W4 and W8. WBC counts increased considerably in Eh0.4 group in W4 and both doses of Eh (Eh0.4 and Eh2.0) groups in W8, while no significant differences were observed between Eh versus control group after injection with bacteria (Fig. 1B).

Statistical analysis showed that the number of lymphocytes in Pg, and Mp2.0 groups were more abundant than the control treatment in W4 ($p < 0.05$). In contrast, no significant differences between treated and control groups were observed in the number of lymphocytes in W8. After challenge with *E. ictaluri*, the quantity of lymphocytes decreased in all treatments, but this value still remained significantly higher in Pg, Mp, Ai0.4, and Eh2.0 groups than in the control treatment (Fig. 2A).

The highest abundance of neutrophils was observed in fish fed Pg0.2 in W4 and in the Mp2.0 group in W8 (Fig. 2B). Although decreasing after the bacterial challenge test, the number of neutrophils was still significantly higher in most extract-supplemented groups compared to control ($p < 0.05$), except for Pa0.2.

Compared to the control, the number of monocytes considerably increased in Pg0.2 and Eh0.4 groups in W4. Similarly, monocytes also increased in fish fed Pa0.2, Mp0.4, and both doses of

Ai compared to control in W8 ($p < 0.05$). After challenge test, the number of monocytes statistically increased in both doses of Pg, Mp and Eh0.4 compared to control group (Fig. 2C).

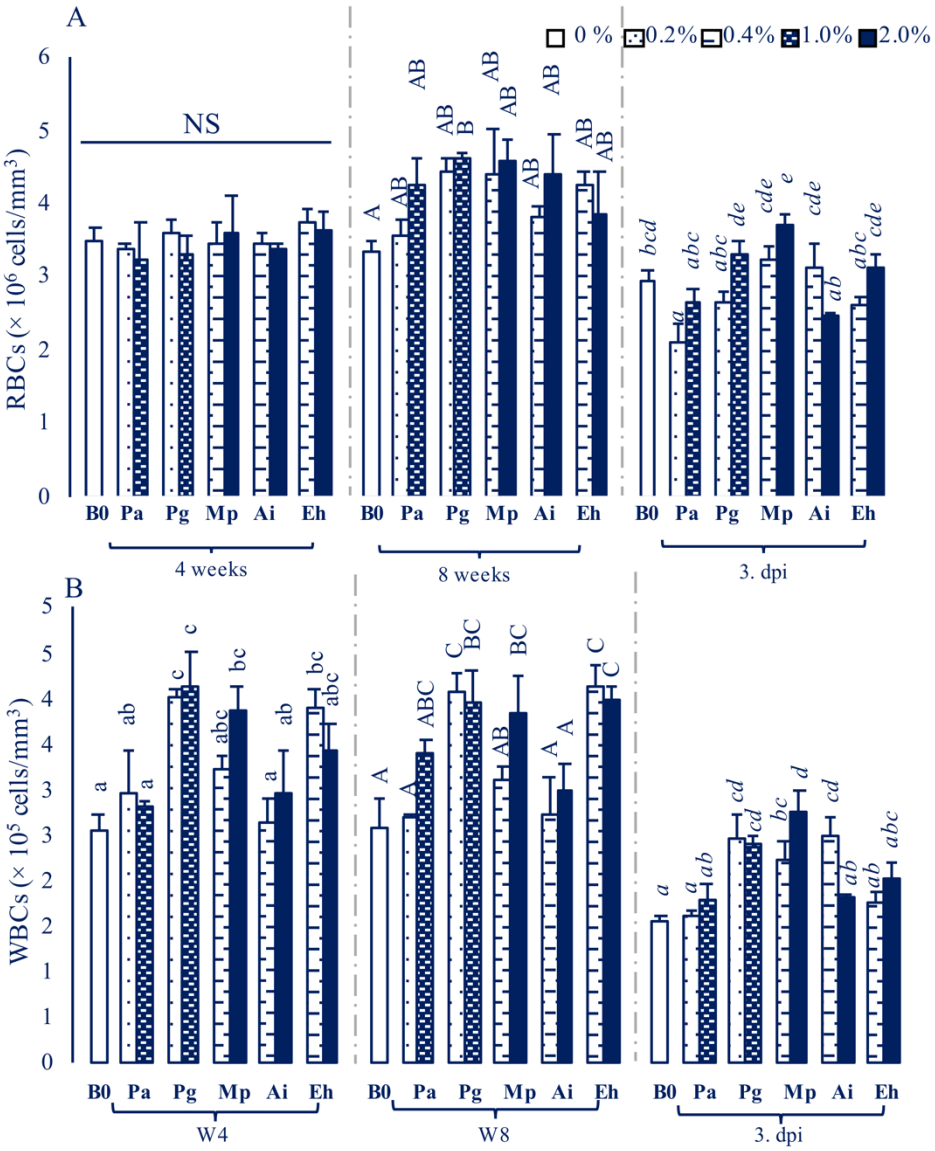

Figure 1. Effects of dietary plant extract administrations on A) total RBCs and B) total WBCs of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$), NS: non-significant.

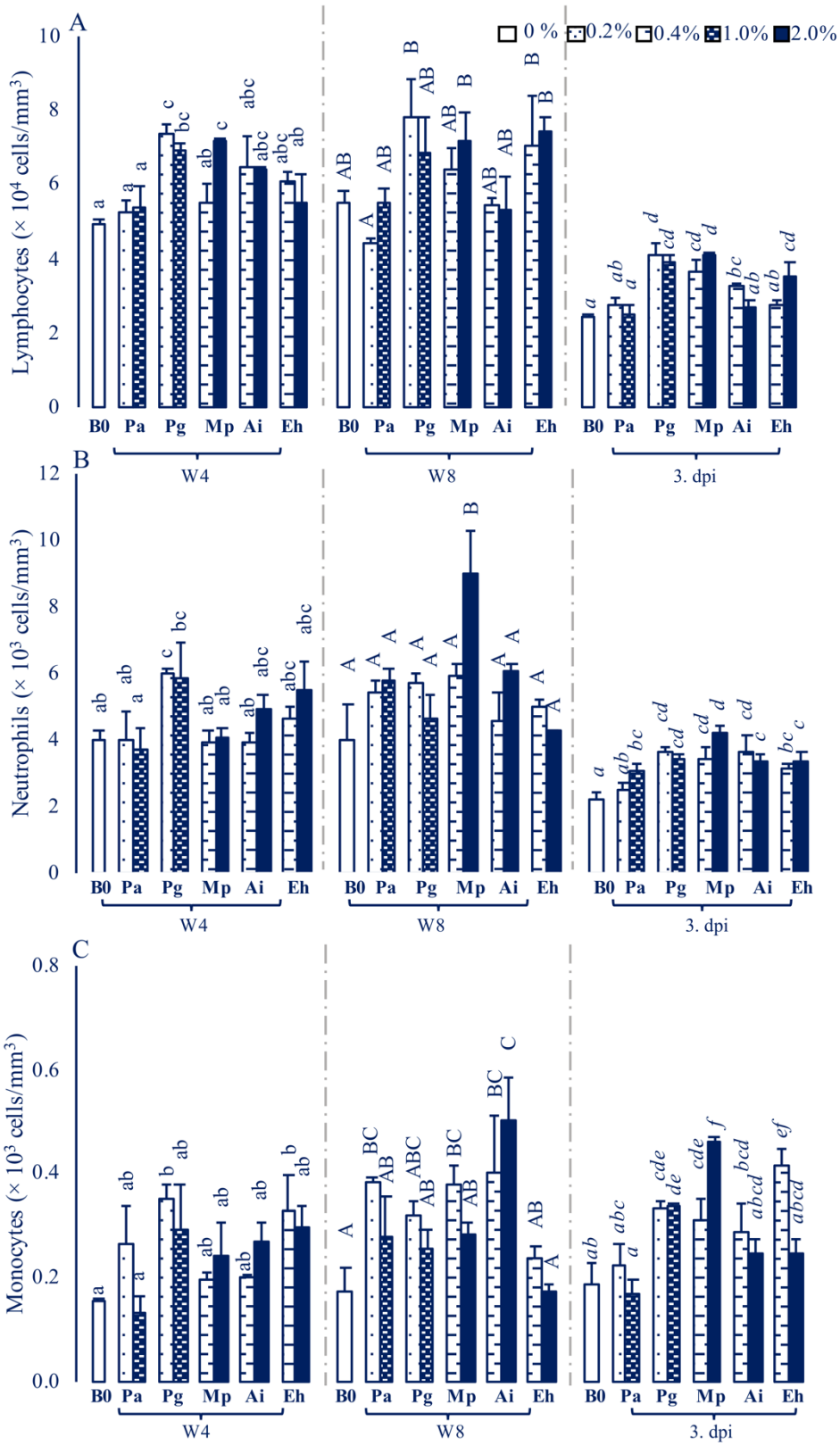

Figure 2. Effects of dietary plant extract administrations on A) lymphocyte, B) neutrophil and C) monocyte numbers of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$).

3.2. Effect of extract supplemented diets on the humoral and mucosal immune response of striped catfish

3.2.1. Lysozyme activity

Plasma lysozyme activity in fish fed Eh (Eh0.4 and Eh2.0) and Ai2.0 was higher than in other groups and significantly differed compared to control in W4 ($p < 0.05$). At the end of the experiment, a significant increase in the plasma lysozyme activity was observed in Pa0.2, Pg0.2, Mp2.0, and Eh2.0 groups. The plasma lysozyme level in most treatments tended to increase after the challenge test. In particular, lysozyme activity in fish fed Pg1.0, Ai (Ai0.4 and Ai2.0), and Eh0.4 diets increased in comparison to the levels in control fish ($p < 0.05$) (Fig. 3A).

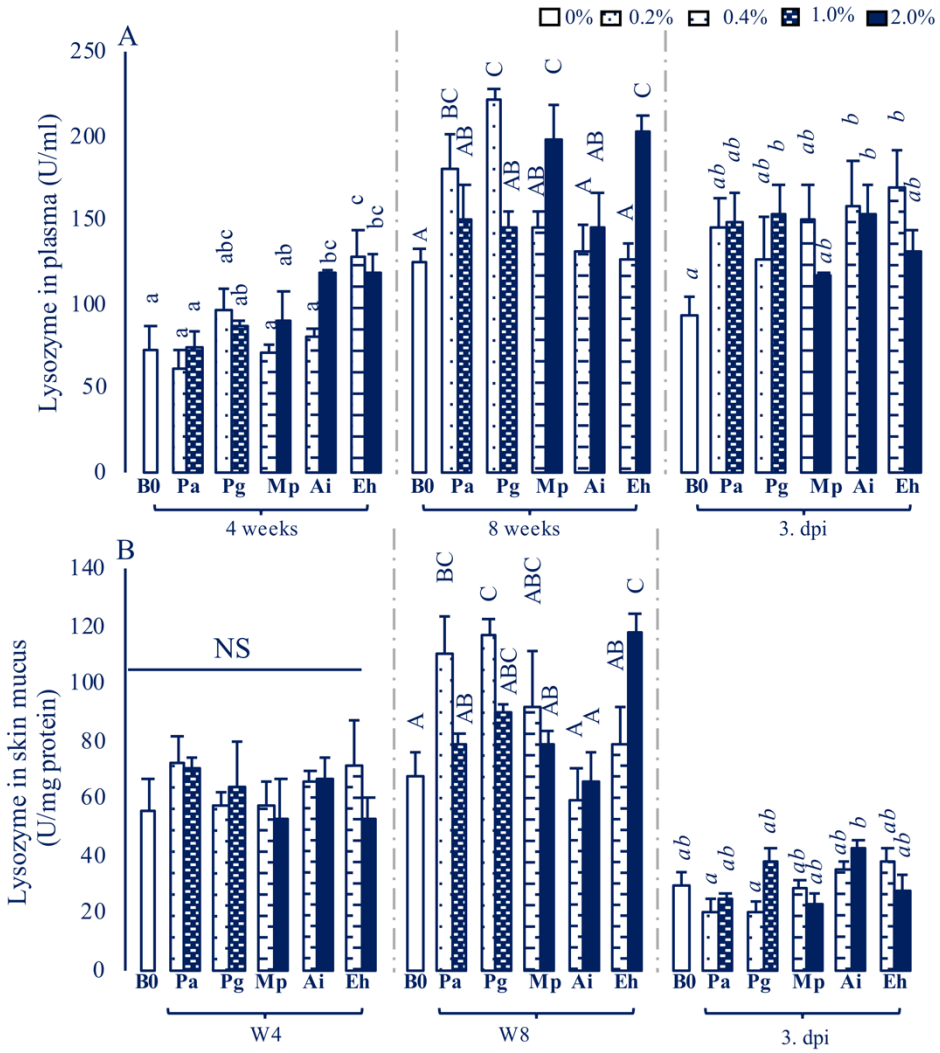

Figure 3. Effects of dietary plant extract administrations on A) plasma and B) skin mucus lysozyme activities of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$). NS: non-significant.

The extract supplemented diets did not influence the lysozyme activity in skin mucus after 4 weeks of feeding (Fig. 3B). However, the lysozyme activity in fish skin mucus increased significantly in Pa0.2, Pg0.2 and Eh2.0 groups in W8. In contrast, the lysozyme activity in skin mucus decreased and did not show any significant difference between extract-enriched diets and the control group after the bacterial challenge test.

3.2.2. Plasma natural hemolytic complement activity

The plasma natural complement activity of striped catfish fed with different experimental diets is shown in Fig. 4. Among the 5 extracts, fish fed with Pa0.2, Pg0.2, and Mp2.0 had a substantially increased plasma natural complement level in W4. At the end of the feeding trial, the ACH50 level was comparatively increased in most of extract groups, which increased relative to the feeding period. Moreover, the ACH50 activity was still higher in Pg and Mp groups compared to other extract groups and statistically different to the control at 3 dpi ($p < 0.05$).

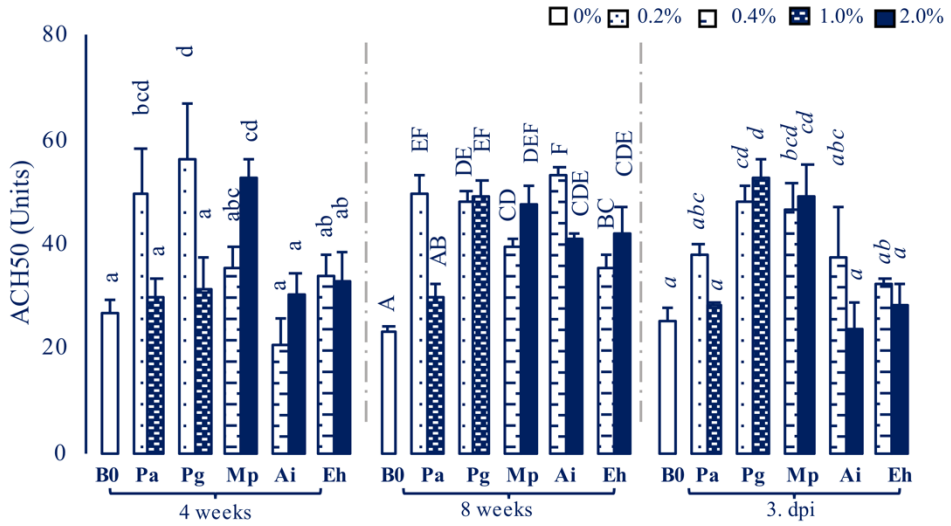

Figure 4. Effects of dietary plant extract administrations on ACH50 activity of striped catfish at different sampling times (W4, W8 and 3. dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$).

3.2.3. Total immunoglobulin

The plasma total Ig started to significantly increase in fish fed diets containing Pa, Mp, Eh, Pg1.0, and Ai2.0 (Fig. 5A) in W4. After 8 weeks of feeding, plasma total Ig was significantly higher in Pa1.0, Mp0.4, and Pg groups than in other groups, while no significant differences in total Ig were observed between treatments after injection with *E. ictaluri*.

The maximum total Ig was recorded in skin mucus of fish fed Pa1.0 compared to other groups in W4. The total Ig significantly increased in Pa, Pg, Mp2.0, and Eh2.0 groups compared to control in W8. Striped catfish fed Pg, Mp2.0, and Eh0.4 supplemented diets showed higher total Ig compared to other diets after the bacterial challenge test (Fig. 5B).

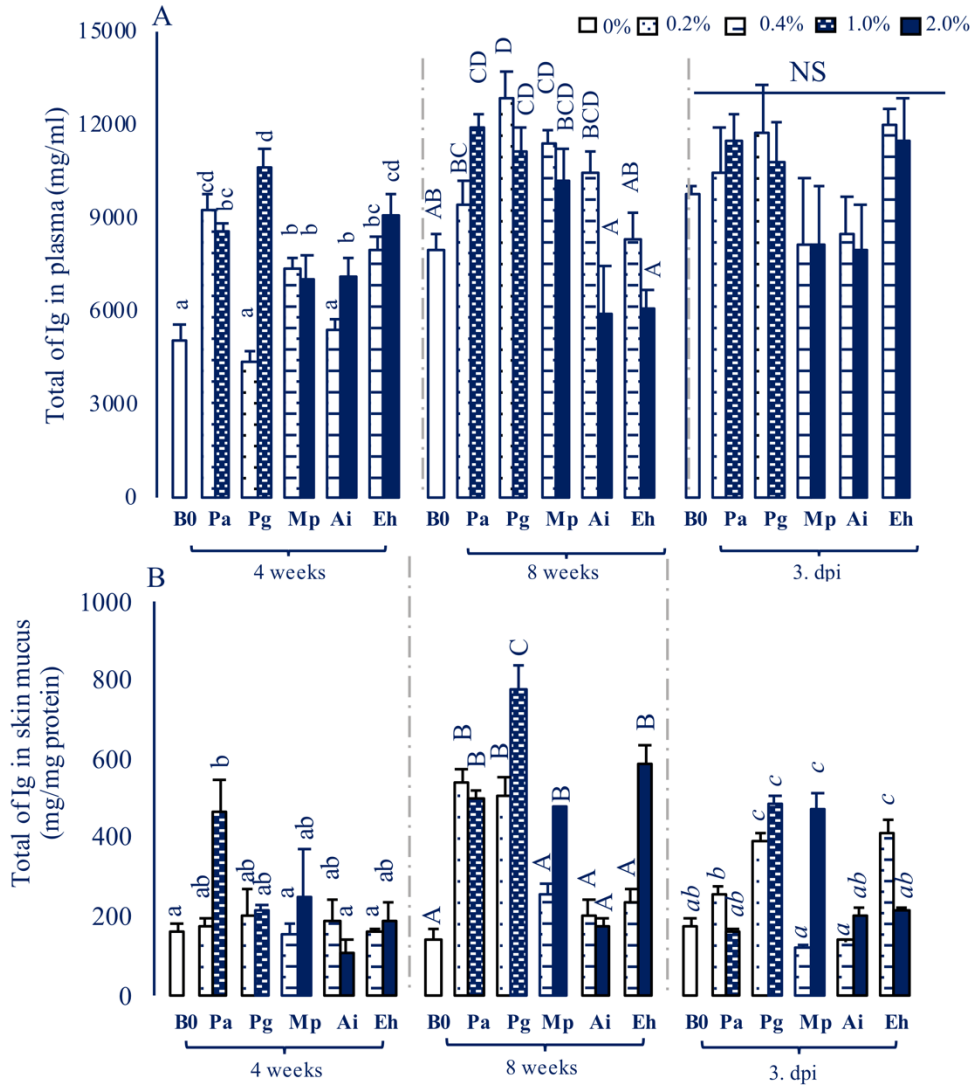

Figure 5. Effects of dietary plant extract administrations on A) plasma and B) skin mucus total immunoglobulin of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$). NS: non-significant.

3.3. Disease resistance of striped catfish against E. ictaluri

Mortality of striped catfish occurred from day 4 to day 8 after infection with *E. ictaluri* (Fig. 6). A maximum of 92.6% mortality rate was recorded in the control group. Mortality was significantly reduced in fish fed plant extract-based diets, especially in Pg, Mp, Pa0.2, Eh2.0, and Ai0.4 groups. Of the different plant extract diets, Mp0.4 and Mp2.0 displayed the highest survival rate post-challenge (58.3% and 55.6%, respectively). No mortalities were recorded in the negative control group. Moreover, bacterial identification found that *E. ictaluri* was detected in all bacterial infection samples (Fig. 7).

The differential effects of striped catfish extract-based diets on immune parameters and resistance to *E. ictaluri* were summarized in table 2.

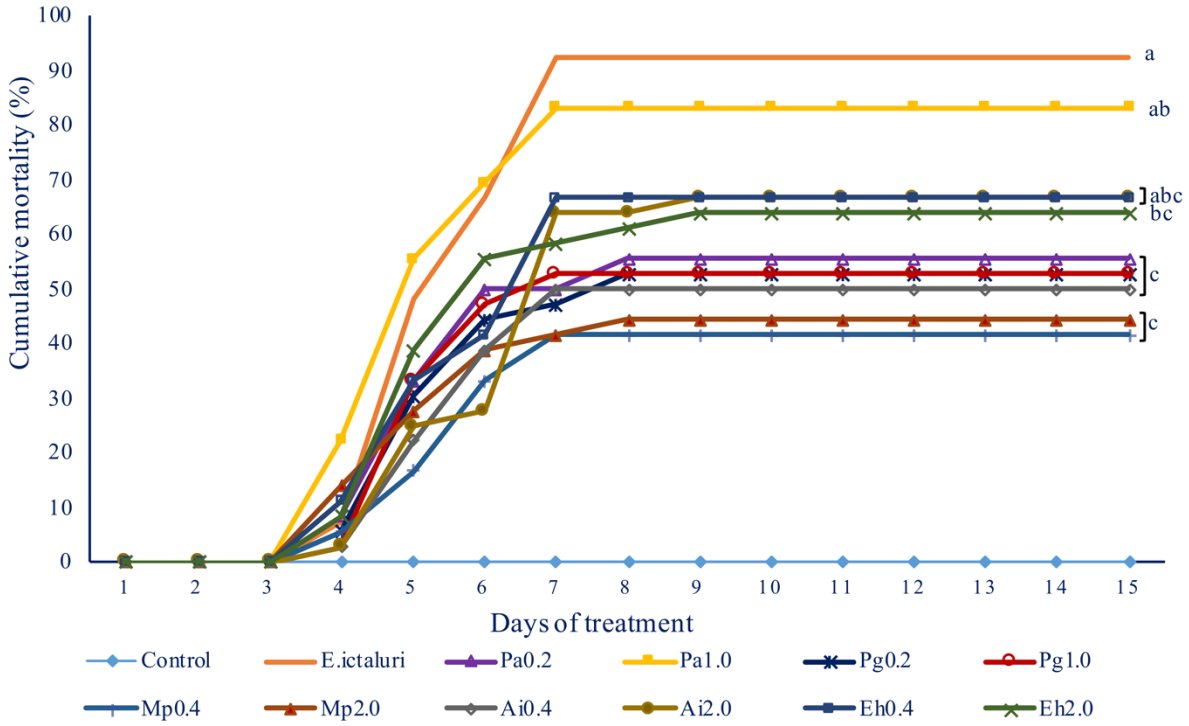

Figure 6. Fish cumulative mortality of challenge test with *E. ictaluri* at W8. Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$).

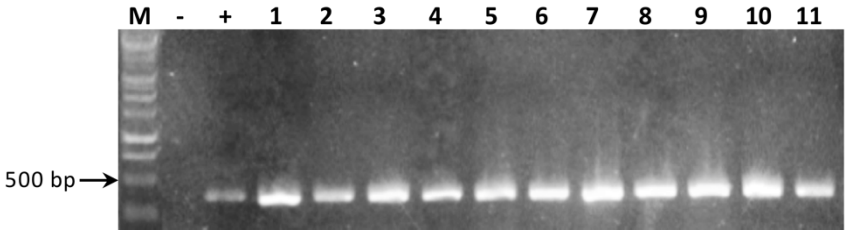

Figure 7. Bacteria confirmation by PCR (M: marker; (−): negative control, (+): positive control, 1–11: fish in each diet were injected with bacteria).

Table 2. Summary results of the analysis of striped catfish immune parameters in short- and long- duration of striped catfish extract-based diets at different

Plant extract	Conc. (%)	Time	Blood parameters					Lysozyme		ACH50	Total Ig		Mortality
			RBCs	WBC	Lymphocytes	Monocytes	Neutrophils	Skin	Serum	Serum	Skin	Serum	
Pa		W4	NS	NS	NS	NS	NS	NS	NS	+	NS	+	
	0.2	W8	NS	NS	NS	+	NS	+	+	+	+	NS	
		3.dpi	NS	NS	NS	NS	NS	NS	NS	NS	+	NS	+
		W4	NS	NS	NS	NS	NS	NS	NS	NS	+	+	
	1.0	W8	NS	NS	NS	NS	NS	NS	NS	NS	+	+	
		3.dpi	NS	NS	NS	NS	+	NS	NS	NS	NS	NS	NS
Pg		W4	NS	+	+	+	+	NS	NS	+	NS	NS	
	0.2	W8	NS	+	NS	NS	NS	+	+	+	+	+	
		3.dpi	NS	+	+	+	+	NS	NS	+	+	NS	+
		W4	NS	+	+	NS	NS	NS	NS	NS	NS	+	
	1.0	W8	+	+	NS	NS	NS	NS	NS	+	+	+	
		3.dpi	NS	+	+	+	+	NS	+	+	+	NS	+
Mp		W4	NS	NS	NS	NS	NS	NS	NS	NS	NS	+	
	0.4	W8	NS	NS	NS	+	NS	NS	NS	+	NS	+	
		3.dpi	NS	+	+	+	+	NS	NS	+	NS	NS	+
		W4	NS	+	+	NS	NS	NS	NS	+	NS	+	
	2.0	W8	NS	+	NS	NS	+	NS	+	+	+	NS	
		3.dpi	+	+	+	+	+	NS	NS	+	+	NS	+

Ai		W4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	0.4	W8	NS	NS	NS	+	NS	NS	NS	+	NS	NS	
		3.dpi	NS	+	+	NS	+	NS	+	NS	NS	NS	+
		W4	NS	NS	NS	NS	NS	NS	+	NS	NS	+	
	2.0	W8	NS	NS	NS	+	NS	NS	NS	+	NS	NS	
		3.dpi	NS	NS	NS	NS	+	NS	+	NS	NS	NS	NS
Eh		W4	NS	+	NS	+	NS	NS	+	NS	NS	+	
	0.4	W8	NS	+	NS	NS	NS	NS	NS	+	NS	NS	
		3.dpi	NS	NS	NS	+	+	NS	+	NS	+	NS	NS
		W4	NS	NS	NS	NS	NS	NS	+	NS	NS	+	
	2.0	W8	NS	+	NS	NS	NS	+	+	+	+	NS	
		3.dpi	NS	NS	+	NS	+	NS	NS	NS	NS	NS	+

+: increase significant level at $p < 0.05$, NS: non-significant. W4: week 4th, W8: week 8th and 3. dpi: three- day post infection; Pa: *Phyllanthus amarus*, Pg: *Psidium guajava*, Mp: *Mimosa pudica*, Ai: *Azadirachta indica*, Eh: *Euphorbia hirta*; RBCs: Red blood cells, WBCs: White blood cells, ACH50: Alternative complement pathway hemolytic

4. Discussion

In the present study, the immunomodulatory effects of different plant extracts were assessed by investigating the changes in immune parameters and disease resistance against *E. ictaluri* in striped catfish. The plant species and extract concentrations used in the study were selected based on a preliminary *in vitro* screening study. In the previous study, we found that *E. hirta, P. amarus*, *P. guajava* and *A. indica* extracts could strongly stimulate some immune markers, while *M. pudica* did not affect the immune responses in head kidney leukocytes of striped catfish after 24 h of *in vitro* culture. Many plants have been reported to possess a wide range of active components such as alkaloids, steroids, phenols, tannins, terpenoids, saponins, glycosides, flavonoids, and many other compounds such as polysaccharides [9, 10]. Moreover, Pa extracts contain three important bioactive constituents (phyllanthin, hypophyllanthin, and corilagin) [45], while guajaverin, quercetin, avicularin, and guavinoside have been found in Pg extracts [46]. Due to the presence of the various compounds and secondary metabolites, the plant species were reported to display immunomodulatory activities [5-8, 47, 48]. Extraction efficiency is partly affected by the type of solvents with varying polarity in the same conditions of pH, temperature as well as extraction time [49, 50]. Total phenol and flavonoid contents of rice paddy-*Limnophila aromatica* (Lamk.) Merr in pure ethanol extract were higher than that in pure acetone, pure methanol and water extracts, although the extraction yield was highest in methanol and decreased following by water, ethanol and acetone [50]. The biological compounds in the extracts including phenols, gallic acid, myricetin were also variable after extraction in different solvents [51]. Moreover, methanol and ethanol were the best solvents for extraction of biological components, which mainly functions in immunostimulatory properties and antibacterial activity [52, 53].

The findings of this study clearly show that oral administration of ethanol extracts could improve fish health status, which was indicated by a higher immune response than in fish fed diets without plant extracts. Hematological parameters are clinical indicators of health and disease conditions [54]. In this study, plant extract-enriched diets did not affect the total RBCs in W4, whereas WBCs, lymphocytes, neutrophils, and monocytes started to increase in a dose dependent manner in fish fed enriched diets from W4. The RBC counts were significantly reduced in Pa groups at 3 dpi compared to controls. This may be due to the presence of higher quantities of tannins and saponins in the ethanolic Pa extract than in other extracts. These compounds have previously been associated with a significant reduction in the number of RBCs [55]. In addition, plants containing polysaccharides usually induce a proliferation of lymphocytes [56-58], possibly explaining the significantly increased number of lymphocytes in striped catfish fed extract-based diets for all sampling times. Among the extracts, diets supplemented with Pg, Mp, and Eh stimulated a significant increase in blood indices in W4, W8, and 3 dpi. A study by Panase et al. [59] demonstrated that WBC numbers were significantly different in hybrid catfish (*Clarias macrocephalus*×*C. gariepinus*) fed Eh leaf extract, although RBCs did not significantly increase on days 30 and 90 of the experiment. Yeganeh et al. [60] also found that RBCs, WBCs, and haemoglobin significantly increased in rainbow trout (*Oncorhynchus mykiss*) after oral diets containing *Spirulina platensis* over

10 weeks. A similar increase in WBCs was reported in common carp when this species was daily dip treated for 10 min over 30 days with an aqueous Ai leaf extract at 1 g/L [61]. The significant increase in the total WBCs after extract stimulation, including various leukocytes such as lymphocytes, monocytes, and neutrophils, could be a good indicator of triggering of striped catfish immunity.

Lysozyme is an important indication of the non-specific humoral immune response. It is primarily released by monocytes and macrophages, and this is higher in macrophages [62]. The present results showed that fish in the groups fed extracts had dose-dependent enhanced plasma lysozyme levels compared to the control throughout the 8-week feeding experiment. Measurement of lysozyme activity is a way to determine whether non-specific immune responses are boosted by immunostimulants. As mentioned earlier, plasma lysozyme level was shown to increase in striped catfish after injection of LPS and levamisole [5, 6]. Similarly, Giri et al. [63] found that the level of plasma lysozyme gene expression significantly increased in juvenile rohu after they were fed Pg supplemented diets for 60 days. The same results were presented by Gobi et al. [16] when tilapias (*Oreochromis mossambicus*) were fed diets containing guava leaf extract for 30 days. Hoseinifar et al. [64] also reported that the enrichment of diets with 0.25% Pg increased the plasma lysozyme activity in common carp fingerlings in an 8-week feeding trial. However, the lysozyme activity does not always increase in fish after being stimulated. Some plant extracts may act as an immunosuppressor of the innate immune system [65, 66]. This may be due to the presence of some phenolic compounds in the plant extracts which could inhibit the enzyme activity. Compounds including flavonoids, for example, strongly bind to lysozyme due to the number and position of hydrogen and type and position of glycosides [67].

Besides the non-specific humoral immune system, lysozyme possesses both bactericidal and opsonin effects that activate the complement system and phagocytes which prevent infectious diseases [62]. In the present study, the plasma complement (ACH50) levels were not significantly enhanced before W4 in most of groups fed with extract-enriched diets, except in Ai2.0 and Eh (Eh0.4 and Eh2.0) groups. In contrast, a statistical increase of ACH50 activity was noted when fish were fed with both doses of Pg, Mp, Ai, Eh, and 0.2% of Pa in W8. This outcome could suggest that the increment of plasma ACH50 activity was time dependent and affected by the length of the extract feeding period. These results are consistent with reports of tilapia fed guava leaf extract-enriched diets, which could significantly enhance ACH50 levels after 60 days of feeding [63]. However, Gobi et al. [16] found that guava leaf extract (0.5 and 1.0 %) could considerably raise the ACH50 activity in tilapia in a shorter 30-day feeding trial. A similar result was also reported by Bahi et al. [68], these authors indicate that dietary administration of fenugreek (*Trigonella foenum graecum*) seeds, alone or combined with *Bacillus licheniformis* (TSB27), *Lactobacillus plantarum*, or *Bacillus subtilis* (B46) strongly enhanced ACH50 activity after 2 and 3 weeks of a feeding experiment. Concerning the fish fed diets supplemented with Pa extracts, the humoral innate immune responses (lysozyme and complement activities) were only found to considerably increase at the lowest level of inclusion. There was lack of increment in lysozyme and complement levels in the groups fed

with Pa at higher concentrations throughout the experimental period. Therefore, boosted immune responses were also affected by the dose of all the extract-supplemented diets.

Aside from the humoral innate immune responses, total immunoglobulin plays an essential role in host defense mechanisms and acts as biomarker for the fish adaptive immune response [69]. In the present study, total Ig significantly increased in extract-enriched diets in both W4 and W8, whereas the total Ig level did not significantly differ between the treatments after injection with *E. ictaluri*. The total Ig activity was also found to be significantly enhanced in koi carp (*Cyprinus carpio koi*) fingerlings after feeding with edible eryngii mushroom powder (*Pleurotus eryngii)* for 63 days [70]. Similar increments of total Ig activity were observed in common carp fed jujube (*Ziziphus jujube*) fruit extract for 8 weeks [34] . Moreover, Laltlanmawia et al. [71] showed that the total Ig content greatly increased for a shorter duration of experiment, 15 and 30 days, when rohu were fed diets supplemented with a mixture of *Withania somnifera* (Ashwagandha) root extract and vitamin C. With regard to Eh extracts, the high and low dose Eh groups had a significantly enhanced total Ig level in W4 compared to control, while Eh did not affect the total Ig after 8 weeks of feeding. Thus, the duration of feeding should also be considered for each plant extract.

Skin is the first physical barrier against the invasion of environmental pathogens in fish [72]. Moreover, skin mucus possesses various cellular (i.e., B, T, and mast cells, macrophages, and granulocytes) and humoral (i.e., complement proteins, immunoglobulins, lectins, lysozyme, proteases, and antimicrobial peptides) immune parameters [73]. In this study, the changes in lysozyme and total Ig were evaluated in the skin mucus of striped catfish. The results showed that dietary administration of plant extracts did not enhance lysozyme activity in skin mucus after 4 weeks, whereas the levels of lysozyme increased significantly in Pa0.2, Pg0.2, and Eh2.0 groups after 8 weeks. In addition, the extract supplemented diets induced stronger effects on total Ig activity of skin mucus in W4, W8, and 3 dpi. In line with our results, Hoseinifar et al. [74] demonstrated that medlar (*Mespilus germanica*) leaf extract stimulated the increase of lysozyme and total Ig activities in skin mucus of common carp after 49 days of a feeding trial. Oral administration of myrtle (*Myrtus communis*) significantly improved mucosal immune responses (the activity of lysozyme, total Ig, and protease) in zebrafish (*Danio rerio*) for 60 days [75]. In contrast, Taee et al. [76] showed no significant differences in skin mucus lysozyme activity between myrtle extract treatments and control groups in rainbow trout during a 60 day feeding period. It can be concluded that treatment with the same immunostimulants may have varying impacts on immune parameters in different animal species.

The elevation of blood parameters in conjunction with the enhanced immune response (humoral and mucosal) possibly contributes to enhancing the defense mechanism against bacterial infection in striped catfish after a plant extract feeding period. The current results revealed that plant extract-based diets significantly increased the survival rates of striped catfish injected with *E. ictaluri* in a dose dependent manner. Mortality was mostly recorded from day 4 to day 8 after the challenge for most treatments. The minimum cumulative mortality in striped catfish was observed in Mp groups at 41.7% and 44.4% respectively. To the best of our knowledge, no publications so far have reported the effects of Mp extract-enriched diets

on disease resistance in aquatic animals. In parallel, Pg extract diets showed significantly improved survival after 8 weeks of feeding. These results are not only comparable but they also confirm earlier studies that reported a significantly reduced mortality rate after *Aeromonas hydrophila* infection in rohu [63, 77] and tilapia [16] fed guava leaf extract-based diets. Moreover, PCR confirmation detected the 16s RNA gene of *E. ictaluri* in most treatments (except the negative control group), suggesting that *E. ictaluri* mainly caused the mortality in striped catfish after the challenge test.

In conclusion, this study confirmed the positive effects of dietary supplementation with plant extracts by improving the immune response of striped catfish. The results demonstrated that both hematological parameters and humoral immune responses of striped catfish were differently enhanced according to the dose and type of extract, as well as to the duration of feeding. Extract dietary administration of *P. guajava* at 0.2 and 1.0%, and *M. pudica* at 2.0% for 8 weeks have the optimal potential for modulating blood parameters and immune responses (humoral and mucosal) in striped catfish, and provided better protection against *E. ictaluri* infection. Further studies are needed to determine the most suitable dose and duration of plant extract treatment in boosting striped catfish immune responses. Moreover, the appetence of the different plant extract-based diets should be investigated, as this can influence feed intake and thus the resulting growth and protection efficiency against pathogens.

Acknowledgments

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Chapter 6

Single or combined dietary supply of *Psidium guajava* and *Phyllanthus amarus* extracts differentially modulate immune responses and liver proteome in striped catfish (*Pangasianodon hyphophthalmus*)

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Hypothesis outlines

After the *in vivo* validation experiment, five extracts including *Euphorbia hirta*, *Phyllanthus amarus*, *Psidium guajava*, *Azadiracha indica*, and *Mimosa pudica* dose-dependently regulated the immune responses and reduced the mortality in striped catfish against the *Edwardsiella ictaluri* infection (Chapter 5). Of the extracts, both doses of *P. guajava* was the best promising plant in activating fish health as well as disease resistance of striped catfish. Another study of PhD student Nguyen Le Anh Dao also documented that *P. amarus* extract induced the highest antioxidant activity in striped catfish after 8-weeks of extract-based diets. The high or low dose of *P. guajava* or *P. amarus* extracts were also the same contribution to the immune response or antioxidant status, respectively. However, it will be spending a lot of time and costs to prepare a larger quantity of extracts when the high dose of plant extracts is applied in the striped catfish farm. In the present study, we are going to find the optimized dose (low dose) of single or mixture supply of *P. guajava* and *P. amarus* extracts to provide better protection of striped catfish health without increasing the costs if applying plant extracts in striped catfish farms. Moreover, the effects of the mixture extract-based diets will also be investigated in order to understand whether the extracts could act in additive or synergic way, or in the opposite way. This study will answer the questions above using the cellular immune and humoral immune biomarkers, as well as bacterial challenge test after 6 weeks post feeding. In addition, liver is one of the most important organs for obtaining an overall assessment of the immune response and antioxidant activity. In the present study, a proteomic approach will be applied to map the regulation of protein expressions from the different pathways related to immune responses and antioxidant activity, providing better knowledge about the mechanism of plant extract-based diets on striped catfish liver.

Abstract

Guava *Psidium guajava* L (Pg) and bhumi amla *Phyllanthus amarus* Schum. et Thonn (Pa) are well-known plants in traditional medicine. However, the capacity of these plants for improving

the immune system of aquatic species has received less attention so far. This study aimed to investigate the effects of single supply or mixture of Pg and Pa extracts on immune responses, disease resistance and liver proteome profiles in striped catfish *Pangasianodon hypophthalmus*. Fish were fed diets including basal diet 0% or one of three doses of each plant extract, either alone or in mixture, 0.08, 0.2 or 0.5% Pg, Pa or mixture (Pg:Pa, v/v) for 6 weeks. The growth was examined at week 6 (W6); the immune parameters (respiratory burst activity (RBA); nitric oxide synthase (NOS), total immunoglobulin, lysozyme and complement activities) were examined at W3, W6 after 6 weeks of feeding and a bacterial challenge test. The challenge test with *E. ictaluri* was done at W6. The liver proteome profiles were analysed in W6 at 0.08% and 0.5% of each extract. The results showed that extract-based diets significantly improved growth parameters in the Pg0.2 group compared to control. The cellular immune responses in spleen and the humoral immune responses in plasma were significantly improved in a dose and time-dependent manner. Diets supplemented with single Pg and Pa extracts, and to lesser extent to combined extracts, could significantly reduce the mortality of striped catfish following *Edwardsiella ictaluri* infection compared to control. The proteomic results indicated that some pathways related to immune responses, antioxidant activities and lipid metabolism were enriched in striped catfish liver after administration of extracts. Several proteins (i.e., CD8B, HSP90AA1, HSP90AB1, *PDIA3*, CASP8, TUBA1C, CCKAR, GNAS, GRIN2D, PLCG1, PRKCA, SLC25A5, VDAC2, ACTN4, GNAI2, LCK, CARD9, NLRP12 and NLRP3) were synergistically upregulated in mixture of Pg and Pa-based diets compared to control and single dietary treatments, suggesting positive synergistic effects of the Pg and Pa combination on immune responses. Taken together, the results revealed that single Pg and Pa extracts at 0.2 and 0.5% and their mixture at 0.08 and 0.5% have the potential to modulate the immune mechanisms and disease resistance of striped catfish, and positively altered the liver proteome profile related to immune system processes.

1. Introduction

Infectious pathogens causing high mortality rates are a major problem leading to tremendous economic losses in aquaculture production worldwide (1). Under aquaculture conditions such pathogens often include viruses, bacteria, parasites and fungi (2). For several years, many approaches have been successfully applied to overcome the obstacles of infectious diseases. The most effective methods in reducing mortality involve strengthening fish defense mechanisms (3). Administration of immunostimulants is considered as a promising alternative therapy, with the aim of replacing antibiotics and chemical use in aquaculture production (4). Increasing numbers of scientific reports are interested in strategies for green growth, with the aim of promoting a more sustainable aquaculture sector. Environmentally-friendly prophylactic and preventive measures using supplementation of natural plant products have been widely developed to enhance the immune system, reduce mortality, and improve growth performance in cultivated animals including fishes. Plant products and their derivatives may contain a variety of biologically active secondary metabolites (5). These compounds possess possible therapeutic value for improving the immune response against infectious diseases. Among plants with potential medical interest, guava *Psidium guajava* (Pg) and bhumi amla

Phyllantus amarus (Pa) are known for their pharmacological activities including anti-bacterial, anti-stress and immune response functions. Active ingredients of Pg and Pa include alkaloids, steroids, phenols, tannins, terpenoids, saponins, glycosides, flavonoids and many other compounds such as polysaccharides (6-9). Moreover, Pg extracts contain guajaverin, quercetin, avicularin and guavinoside (8), while three important bioactive components (phyllanthin, hypophyllanthin and corilagin) have been found in Pa extracts (10). Earlier studies reported that dietary supplementation with Pg extracts significantly improved the growth performance, antioxidant and immune parameters in rohu *Labeo rohita* (11, 12), Mozambique tilapia *Oreochromis mossambicus* (13) and common carp *Cyprinus carpio* (14). To the best of our knowledge, there are a limited number of publications so far on the effects of Pa extract-enriched diets on immune responses of aquatic species. Only the study by Sundaram et al. (2016) (15) has demonstrated that acetone and petroleum ether extracts of Pa could protect the freshwater crab *Paratelphusa hydrodomous* against white spot syndrome virus. Pa extracts have been demonstrated to exert anti-inflammatory effects by downregulating prospective inflammatory signalling mediators in mammals (10, 16). 70% ethanol extracts of Pa were also reported to induce apoptosis in a hepatocellular carcinoma HepG2 cell line *in vitro*, with an increased activity of caspases 3 and 7 (17).

The immune system is a complex defense mechanism of the body, comprising balanced multicellular and physiological mechanisms. The teleost immune system is weaker than that of mammals due to their lack of bone marrow, lymph nodes and germinal centers (18). The innate immune response is a first line defense mechanism to eliminate invading pathogens (*e.g.* bacteria, viruses, fungi, protozoans and parasites). Skin mucus is well known as a primary physical barrier against foreign substances that have adhered to the fish's body surface. Moreover, phagocytes (macrophages and monocytes), neutrophils, dendritic cells and natural killer cells are various cell types governing the innate immune response. Physical properties of the innate immune response including lysozymes, complement, lectins, transferrin, agglutinins and lysine act as biological defense molecules in fish. Additionally, adaptive immunity is mediated by two lymphocyte populations classified as T cells and B cells. Immunoglobulins, T-cell receptors and major histocompatibility complex (MHC) class I or/and II are molecules involved in adaptive immunity. Teleost fish exert immune responses against a variety of antigens with specificity and memory. In aquaculture, the elevation of immune parameters is a useful tool to assess whether immunostimulants affect the fish immune response. However, traditional physiological and biochemical analyses alone cannot determine in depth the pathways of molecular mechanisms involved in the action of immunostimulants, such as various plant extracts. In recent years, proteomic approaches have been widely developed as a powerful tool to interpret specific influences of exercise and diet on the metabolic process of the immune system (19). In vertebrates, the liver represents an important organ for metabolism, nutrient storage and immune responses by producing cytokines, chemokines and complement components (20). By using a quantitative proteomics approach, Mendez et al. (2017) demonstrated the capacity of high-fat and sucrose-based diets to enhance fatty acid beta-oxidation, insulin signalling, ameliorate endoplasmic reticulum stress and protein oxidation

pathways in rat liver (21). Moreover, several pathways including D-arginine and D-ornithine metabolism, MAPK signalling, Wnt signalling and gap junction pathways were significantly enhanced in obscure pufferfish *Takifugu fasciatus* liver under low-temperature stress (22). Causey et al. (2018) reported in rainbow trout *Oncorhynchus mykiss* that the immune function and liver metabolism promoted rewiring in host defense responses during infection with *Aeromonas salmonicida* (23). Semi-synthetic diets enriched with different sources of proteins including soybean, fish, chicken, pork or beef altered the metabolism in rat liver, resulting in mediated antioxidant and anti-inflammatory responses (24). With regard to diets supplemented with plant extracts, no publications so far have addressed the link between protein expression levels and plant extracts in fish liver.

After a large screening study to test the *in vitro* capacity of 20 plant extracts to enhance the immunity of striped catfish *Pangasianodon hypophthalmus* (25), we previously found that five ethanol plant extracts including Pa, Pg, sensitive plant *Mimosa pudica* L., neem *Azadirachta indica* A. Juss and asthma plant *Euphorbia hirta* L. were potentially interesting for modulating blood parameters, immune responses and providing better protection to striped catfish against infection with the pathogenic bacteria *Edwardsiella ictaluri* after 8 weeks of feeding (26). In the present study, we evaluated the effects of different dietary doses of Pg and Pa (alone or in combination) on growth performance, immune parameters and disease resistance against *E. ictaluri* in striped catfish. In addition, a gel-free proteomic analysis was carried out using the fish liver to better understand the metabolic pathways involved when the selected plant extracts, alone or in combination, were added to the diet of striped catfish.

2. Materials and methods

2.1. Diet preparation

Ethanol extracts from Pg and Pa were obtained as previously described (26). The experimental diets were prepared using the extract ingredients to contain 0%; 0.08%, 0.2% and 0.5% of each Pg or Pa extract and their mixture (v: v) at similar concentrations (Table 1). The basal and experimental diets were prepared following previous study (26) and pellets of 2 mm were stored at −20 °C until use.

2.2. Fish

Farm-raised striped catfish juveniles (15-20 g) obtained from a local fish farm in Vinh Long province of Vietnam were transported to the laboratory in plastic bags filled with oxygenated water. The fish were acclimatized to laboratory conditions for 15 days and then stocked into composite tanks (250 L) in a flow through freshwater supply system, and fed twice a day with the formulated diet at a rate of 2% of their body weight/day.

Table 1. Composition of experimental diets

Ingredients (100 g of feed)	**Control diet**	**Experimental diets**								
		Pg0.08	**Pg0.2**	**Pg0.5**	**Pa0.08**	**Pa0.2**	**Pa0.5**	**Mix0.08**	**Mix0.2**	**Mix0.5**
[a]Soybean meal (g)	24	24	24	24	24	24	24	24	24	24
[b]Rice bran (g)	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5
[c]Casava (g)	17.96	17.88	17.76	17.46	17.88	17.76	17.46	17.80	17.56	16.96
[d]Fishmeal (g)	24	24	24	24	24	24	24	24	24	24
[e]Fish oil (g)	1	1	1	1	1	1	1	1	1	1
[f]Premix* (g)	3	3	3	3	3	3	3	3	3	3
Phytase	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
[g]Gelatin (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
[h]Butylated hydroxytoluene (BHT)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Plant extracts (g)										
Pg	–	0.08	0.2	0.5	–	–	–	–	–	–
Pa	–	–	–	–	0.08	0.2	0.5	–	–	–
Mixture	–	–	–	–	–	–	–	0.08	0.2	0.5

Pa: *Phyllanthus amarus*, Pg: *Psidium guajava*, Mp: *Mimosa pudica*, Ai: *Azadirachta indica*, Eh: *Euphorbia hirta*

[a]Wilpromil R Soy Protein Concentrate, Yihai (Fangchenggang) Soybeans Industries, (Wilmar Group), Fangchenggang, China.

[b]Cai Lan Oils & Fats Industries Company, Can Tho Branch, Can Tho City, Vietnam.

[c]Hong Ha Company, Can Tho City, Vietnam.

[d]Minh Tam, Can Tho, Vietnam.

[e]Vegetable oil (Simply, Vietnam) and squid oil (Vemedim, Vietnam) at a ratio of 1:1.

[f]The vitamin/mineral premix (Unit/kg) from Vemedim, Can Tho, VietNam: vitamin A, 6000 IU; vitamin D3, 5600 IU; vitamin E, 160 IU; vitamin B1, 10 mg; vitamin B6, 20 mg; vitamin B12, 0.03 mg; vitamin K, 0.3 mg; riboflavin, 60 mg; vitamin C, 300 mg; pantothenic acid, 60 mg; folic acid, 8 mg; nicotinic acid, 184 mg; biotin, 0.3 mg; iron, 50 mg; copper, 10 mg; iodine, 9 mg; zinc, 34 mg; selenium, 0.4 mg; manganese, 30 mg

[g]Xilong Chemical Industry Incorporated (China)

[h] Honshu Chemical Industry Company, Japan

2.3. Bacteria preparation

E. ictaluri were cultured on tryptic soy agar plates (TSA, Merck) for 48 h at 28°C following the previous study by Hang et al. (27). Then, a single colony was collected and harvested into tryptic soy broth (TSB, Merck). This suspension was shaken overnight, at 180 rpm at 28°C. Then, bacteria were centrifuged at 5000 rpm at 4°C for 5 min and washed 3 times with 0.85% NaCl solution. The mean colony count used the optical density method (28) and OD was adjusted to a value of 0.1 by spectrophotometer (Thermospectronic, USA) at 590 nm. Then, this suspension was diluted 1000 times with NaCl solution before injection into the fish.

2.4. Experimental design

Fish were randomly distributed into ten distinct treatments, each treatment in triplicate. Fish were fed the experimental diets described above for 6 weeks, at 2% of body weight and three times (8 am, 12 am, and 5 pm) daily. Tank capacity was 250 L, each tank contained 45 fish. Water temperature, dissolved oxygen and pH were monitored daily and maintained at 30 ± 2°C, 5.7 ± 0.01 mg L^{-1}, 7.5 ± 0.02, respectively, throughout the experimental period. After 6 weeks of feeding, all groups (45 fish/group) fed plant extract-based diets were injected intraperitoneally with 0.1 mL LD50 (1×10^7 CFU/mL) of *E. ictaluri* suspension. At the same time, the control groups were divided into two small groups, the first one was the control injected with 0.1 mL of 0.85% NaCl solution and the second one challenged with 0.1 mL LD50 of *E. ictaluri*. All groups were maintained in triplicate, 15 fish per tank. Cumulative mortality was recorded daily for 14 days after the challenge test. In order to be sure that the mortalities were due to the bacterial infection, *E. ictaluri* was re-isolated and identified by PCR confirmation as described in (27). The daily feed supplied was recorded, and the uneaten feed was collected 1 h after feeding by syphoning, followed by drying, weighing, and finally subtracted from the total amount of supplied feed to calculate the actual feed intake.

2.5. Sample collection

The skin mucus samples were collected at W3 and W6 of the feeding trial, and 3 days post injection (3 dpi) according to Ross et al. (29) with slight modifications. Briefly, 3 fish per tank were randomly collected and anesthetized using 0.1 ppm of M222 (Sigma-Aldrich, USA). Fish surfaces were individually washed with distilled water and then transferred into polyethylene bags containing 1 mL of PBS 1X. After 2 min with gentle shaking, mucus was collected, transferred to 2.0 mL sterile Eppendorf tubes and centrifuged ($1500 \times g$ for 10 min at 4°C). The supernatant was stored at –80°C for further analysis. Similarly, blood samples were obtained from the caudal vein of individual fish (9 fish per treatment, 3 fish per tank) and centrifuged at 4000 rpm for 10 min. The plasma supernatant was collected into new Eppendorf tubes and kept at –80°C until analysis. At the end of the feeding trial, all experimental fish were weighed for growth performance calculations.

2.6. Growth performance

All fish were deprived of food 24 h before weighing and sampling, and the following parameters were measured at the end of feeding trial (6 weeks):

Weight gain (WG) = 100 × (W2 – W1) / W1

Specific growth rate (SGR) = 100 × (ln W2 – ln W1) / T

Feed conversion ratio (FCR) = feed intake (g) / weight gain (g)

Where W1 is the initial weight (gram), W2 is the final weight (gram) and T is the number of days in the feeding period.

2.7. Cellular immune variables

2.7.1. Spleen respiratory burst assay

Respiratory burst was adapted from Rock et al. (30). Spleens were weighed and then mashed in L-15 medium (Saint Louis, MO, USA) through a 100 µM nylon mesh. Cell suspensions were washed and centrifuge (1000 × *g*, 5 min, 28°C) twice in L-15 medium. The culture media were then replaced by the corresponding fresh culture media containing 2 mg ml^{-1} nitroblue tetrazolium (NBT). Cells were incubated for 1 h at 28°C in a light protected environment. After 1 h, the cells were washed twice in PBS and the reaction was stopped by adding 200 µL of methanol. The cells were rinsed by centrifugation (1000 × *g*, 10 min, 4°C) and finally air dried for 10 min. Resulting formazan was dissolved in 240 µL of KOH 2M and 280 µL of N-dimethylformamide. The absorbance of the final supernatant was measured at 550 nm. A standard curve was produced using serial dilutions of NBT directly dissolved in KOH 2M and N-dimethylformamide. Samples and negative control without cells were performed in duplicate. Activity was reported on protein concentration in spleen measured by Bradford assay.

2.7.2. Spleen nitric oxide species assay (NOS)

Production of NOS was measured by the Griess reaction. First, 100 µL of cell suspensions collected from the spleen were incubated with 5 µL of *E. ictaluri* suspension (OD 2) resuspended in corresponding culture media for 1 h at 28°C. Then, 100 µL of Griess reactant was added and solutions were incubated for 15 min. The absorbance was measured at 540 nm. A standard curve was produced by using serial dilutions of $NaNO_3$. Negative control corresponded to culture media (without cells) incubated with *E. ictaluri* suspension without cells and Griess reactant. Activity was reported for protein concentration in spleen measured by Bradford assay.

2.8. Humoral immune variables

2.8.1. Lysozyme assay

The lysozyme assay protocol was adapted from Ellis (31) and Milla et al. (32). In 96–well microplates, the lysozyme activity assay was initiated by mixing 10 µL of plasma or 20 µL of skin mucus with 130 µL of lyophilised *Micrococcus lysodeikticus* (Sigma) suspension in phosphate buffer, pH 6.2 (0.6 mg mL^{-1} for plasma and 0.3 mg mL^{-1} for skin mucus). The difference in absorbance at 450 nm was monitored between 0 and 30 min for plasma (0 and 15 min for the skin) and used to calculate lysozyme activity in units. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

2.8.2. Complement assay

The plasma alternative complement pathway was assayed using rabbit red blood cells (RRBC, Biomerieux, Craponne, France) as targets following Sunyer and Tort (33) and adapted by Milla et al. (32). Briefly, 10 µL of RRBC suspension (3%) diluted in veronal buffer (Biomerieux) was mixed with serial dilutions of plasma (60 µL total volume). After incubation for 100 min at 28°C, the samples were centrifuged at 2000 × *g* for 10 min at room temperature. The spontaneous hemolysis was obtained by adding 60 µL of veronal buffer to 10 µL of RRBC. The total lysis was obtained by adding 60 µL of distilled water to RRBC. The absorbance was measured at 405 nm. Appropriate calculations served to estimate complement activity.

2.8.3. Total Ig assay

The total immunoglobulin concentration the sample was measured using the method of Siwicki and Anderson (34), modified by Milla et al. (32). Briefly, immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, Sigma). Plasma or skin mucus samples were mixed with 12% PEG solution (v:v) for 2 h at room temperature under constant shaking. After centrifugation at 1000 × *g* for 10 min, the supernatant was collected and assayed for its protein concentration. The total immunoglobulin concentration was calculated by subtracting this value from the total protein concentration in the plasma before precipitation with PEG.

2.9. Statistical analyses

All statistical analyses were performed using SPSS version 20. Results are presented as means ± SEM (standard error of the mean). The normality of the data and the homogeneity of variance between groups were tested using Shapiro-Wilks and Levene tests. One-way analyses of variance (ANOVA) and Duncan's multiple range test at a confidence level of 95% ($p < 0.05$) were used to determine significant differences between immunological variables in fish from the different plant extract treatments and control treatment.

2.10. Liver quantitative proteomic analysis

2.10.1. Protein extraction and digestion

Proteomic analysis was performed on liver samples at low (0.08%) and high (0.5%) doses of each kind of plant extract in W6. Three fish from the same tank were pooled and tanks were considered as independent biological replicates. Briefly, samples were ground to powder and the powder was dissolved in lysis buffer [8 M urea and 40 mM tris-HCL or triethylammonium bicarbonate (TEAB; pH 8.5) supplemented with 1 mM phenylmethylsulfonyl fluoride and 2 mM ethylenediaminetetraacetic acid to a final concentration of 10 mM, and the suspension was sonicated at 200 W for 1 min prior to centrifugation at 25,000 × *g* for 20 min at 4°C. Proteins were reduced using 5 mM DTT (dithiothreitol) and alkylated using 15 mM iodoacetamide. Proteolysis was performed with 0.5 µg of trypsin and allowed to continue overnight at 37°C. Each sample was dried under vacuum using a Savant Speedvac Concentrator.

2.10.2. LC-IMS (ion mobility separation)-QTOF-MS analysis (HDMSE)

Peptide separation using nanoUPLC

Before peptide separation, the samples were dissolved in 20 µL of 0.1% (v/v) formic acid and 2% (v/v) acetonitrile (ACN). The peptide mixture was separated by reverse phase chromatography on a NanoACQUITY UPLC MClass system (Waters, MA, USA) working with MassLynx V4.1 (Waters, MA, USA) software. 200 ng of digested proteins were injected on a trap C18, 100 Å 5 µm, 180 µm × 20 mm column (Waters, MA, USA) and desalted using isocratic conditions with a flow rate of 15 µL/min using a 99% formic acid and 1% (v/v) ACN buffer for 3 min. The peptide mixture was subjected to reverse phase chromatography on a C18, 100 Å 1.8 µm, 75 µm × 150 mm column (Waters, MA, USA) PepMap for 120 min at 35°C and a flow rate of 300 nL/min using a two part linear gradient from 1% (v/v) ACN, 0.1% formic acid to 35% (v/v) ACN, 0.1% formic acid, and from 35% (v/v) ACN, 0.1% formic acid to 85% (v/v)) ACN, 0.1% formic acid. The column was re-equilibrated to initial conditions after washing for 10 min at 85% (v/v) ACN, 0.1% formic acid at a flow rate of 300 nL/min. For online LC-MS analysis, the nanoUPLC was coupled to the mass spectrometer through a nano-electrospray ionisation (nanoESI) source emitter.

IMS-HDMS^E (ion mobility separation-high definition enhanced) analyses were performed on a SYNAPT G2-Si high definition mass spectrometer (Waters, MA, USA) equipped with a NanoLockSpray dual electrospray ion source (Waters, MA, USA). Precut fused silica PicoTip^R emitters for nanoelectrospray, outer diameter: 360 µm; inner diameter: 20 µm; 10 µm tip; 2.5 inches length (Waters) were used for samples and precut fused silica TicoTip^R emitters for nanoelectrospray, outer diameters: 360 µm; inner diameter: 20 µm; 2.5 inches length (Waters, MA, USA) were used for the lock mass solution. The eluent was sprayed at a spray voltage of 2.4 kV with a sampling cone voltage of 25 V and a source offset of 30 V. The source temperature was set to 80°C. The HDMS^E method in resolution mode was used to collect data from 15 min after injection to 106 min. This method acquires MS^E in positive and resolution mode over the m/z range from 50 to 2000 with a scan time of 1 sec. with a collision energy ramp starting from ion mobility bin 20 (20 eV) to 110 (45 eV). The collision energy in the transfer cell for low-energy MS mode was set to 4 eV. For the post-acquisition lock mass correction of the data in the MS method, the doubly charged monoisotopic ion of [Glu^1]-fibrinopeptide B was used at 100 fmol/µL using the reference sprayer of the nanoESI source with a frequency of 30 s at 0.5 µL/min into the mass spectrometer.

ESI-QTOF data processing

HDMS^E data were processed with Progenesis QI (Nonlinear DYNAMICS, Waters) software using a Uniprot Pangasius sequence protein database (193970 entries). Propionamide as the fixed cysteine modification, oxidation as the variable methionine modification, and trypsin as the digestion enzyme were selected and one miss cleavage was allowed. Three biological and three technical replicates were used for each sample. The non-conflicting method was used as the relative quantification method. To identify statistically significant differentially expressed proteins (DEPs), the t-tests were adopted and proteins with at least 1.2-fold change ratio and a p-value < 0.05.

2.10.3. Bioinformatics analysis

The functional background annotation analyses for all the identified proteins were mapped with Gene Ontology (GO) terms (http://geneontology.org/). The differentially expressed proteins (DEPs) were also subjected to the functional categorisation of the GO Terms tool, applying the hypergeometric statistical test with correction by the Bonferroni method, considering p-value < 0.05 as significant. At least five proteins in each GO term focusing on immune system processes were considered as the interaction network. The enrichment analysis of statistically significant DEPs for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was performed using the database for annotation, visualization and integrated discovery (DAVID) Bioinformatics Resource 6.8 (35) and STRING (Search Tool for the Retrieval of Interacting Genes) software (v.11.0) (http://string-db.org). At least three proteins related to the immune system in each KEGG with a requisite minimum confidence score of 0.4 were considered to be a significantly enriched pathway.

3. Results

3.1. Growth performance

The effects of extract-based diets on the growth performance of *striped catfish* are shown in Table 2. At the end of the feeding trial, WG and SGR in the Pg0.2-based treatment were significantly increased compared to Pg0.08 and control diets ($p < 0.05$). The FCR values were statistically lower in fish fed Pg0.2 and Pa0.2 diets than in fish of the control treatment.

Table 2. Effects of dietary administration of single versus combination of *P. guajava* and *P. amarus* extracts on growth performance and feed utilization of *P. hypophthalmus* in W6.

Name	Concentration (%)	WG (%)	SGR (%)	FCR
Control	0.00	56.01 ± 11.9^{a}	1.05 ± 0.18^{a}	2.31 ± 0.49^{a}
P. guajava	0.08	59.94 ± 9.83^{a}	1.11 ± 0.14^{a}	2.14 ± 0.38^{ab}
	0.20	97.92 ± 31.5^{b}	1.60 ± 0.39^{b}	1.40 ± 0.53^{c}
	0.50	68.20 ± 6.25^{ab}	1.23 ± 0.08^{ab}	1.85 ± 0.17^{abc}
P. amarus	0.08	76.44 ± 12.2^{ab}	1.34 ± 0.16^{ab}	1.67 ± 0.28^{abc}
	0.20	82.64 ± 14.2^{ab}	1.42 ± 0.18^{ab}	1.55 ± 0.29^{bc}
	0.50	81.16 ± 15.4^{ab}	1.40 ± 0.20^{ab}	1.59 ± 0.31^{abc}
Mixture	0.08	75.72 ± 21.2^{ab}	1.32 ± 0.30^{ab}	1.77 ± 0.59^{abc}
	0.20	75.01 ± 10.8^{ab}	1.32 ± 0.14^{ab}	1.70 ± 0.23^{abc}
	0.50	78.67 ± 7.42^{ab}	1.38 ± 0.10^{ab}	1.61 ± 0.16^{abc}

Values (Mean ± SEM, n=3) with different superscript in the same column are significantly different ($P<0.05$). WG: Weight gain rate; SGR: Specific growth rate; FCR: Feed conversion ratio.

3.2. Spleen respiratory burst activity and nitric oxide synthase production

The Pa0.2-based diet significantly increased RBA levels compared to control in W3 ($p < 0.05$), while the levels were not statistically different between experimental and control diets in W6. (Fig. 1A). However, the levels of RBA considerably increased in diets supplemented with

Pa0.08, Pa0.2 and Mix (all doses) compared to Pg and control diets after injection with *E. ictaluri* ($p < 0.05$).

Single versus combination of Pg and Pa-enriched diets did not affect the NOS level compared to control in W3, although the NOS abundance in Pa0.08 was significantly lower than that of the Mix0.2 treatment ($p < 0.05$). Significantly lower levels of NOS were observed in Pg0.2-based diet compared to control in W6. (Fig. 2B). The NOS activity statistically increased only in the Pg0.2-based diet compared to control after bacterial challenge ($p < 0.05$).

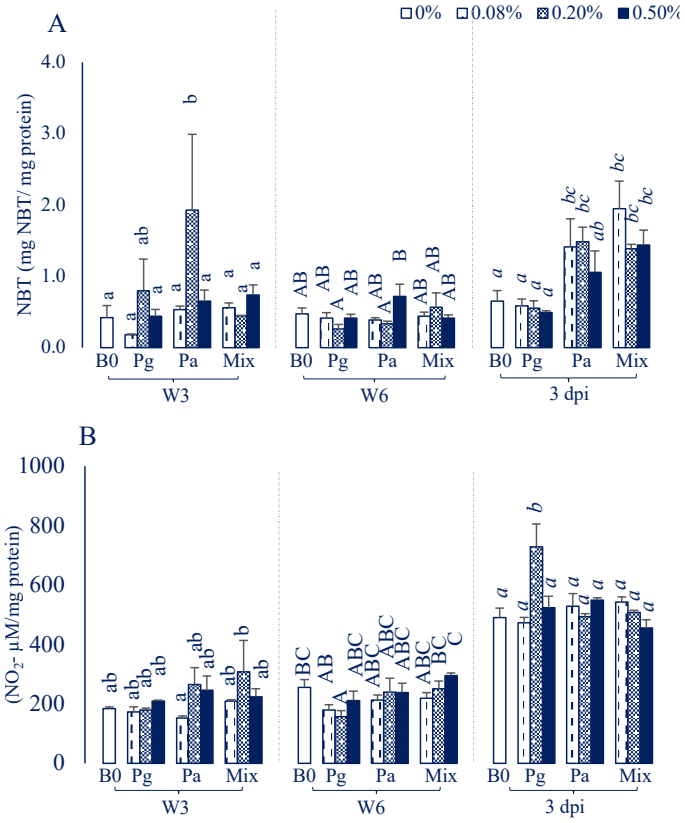

Figure 1. A) Respiratory burst activity (RBA) and B) Nitric oxide synthase (NOS) production activity in striped catfish fed extract-based diets containing a single supply or mixture of *P. guajava* and *P.* amarus at 0; 0.08; 0.2 and 0.5% after 3, 6 weeks and 3 dpi. B0: basal diet; Pg: *P. guajava*; Pa: *P. amarus;* Mix: Mixture of P. *guajava* and *P. amarus*; W3: week 3; W6: week 6; 3 dpi: three days post injection with *E. ictaluri*. Different letters indicate differences among diets at a given time point ($p< 0.05$). Values are means ± SEM, n = 3.

3.3. Humoral and mucosal immune responses

3.3.1. Lysozyme activity

The plasma lysozyme activity was significantly influenced by the extract-based diet groups (Fig. 2A). In particular, the lysozyme levels were significantly increased in most of the treatments supplemented with extracts in W3, except Pg0.08 group. After 6 weeks of feeding, the plasma lysozyme activity was recorded to significantly increase in fish fed Pg, Pa0.2 and Mix0.5 ($p < 0.05$). The lysozyme level was observed to reach a maximum peak in Pg0.2 extract-based diet fed fish after intracellular injection with *E. ictaluri*, while there was a

significant decrease in lysozyme levels in the three concentrations of Mix compared to control.

In parallel, the lysozyme activities in skin mucus were also significantly increased in Pa0.08 and Mix diets in W3 (Fig. 2B). However, the lysozyme levels considerably increased in Pg0.2, Pa0.2 and Mix0.2 compared to control ($p < 0.05$) in W6. At 3 dpi, the lysozyme levels were significantly higher in Pg0.5, Pa0.08 and Mix (all doses) compared to control treatment ($p < 0.05$), while Pa0.5 significantly inhibited lysozyme activity compared to control.

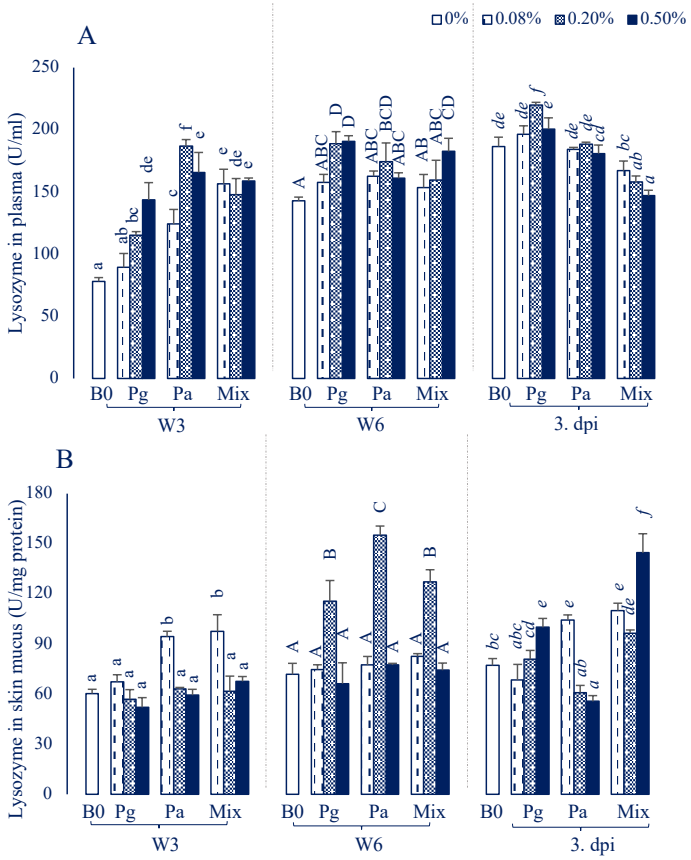

Figure 2. Lysozyme activity in A) plasma and B) skin mucus of striped catfish fed extract-based diets supplemented with a single supply or a mixture of *P. guajava* and *P. amarus* at 0; 0.08; 0.2 and 0.5% after 3, 6 weeks and 3 dpi. B0: basal diet; Pg: *P. guajava*; Pa: *P. amarus;* Mix: Mixture of P. *guajava* and *P. amarus*; W3: week 3; W6: week 6; 3 dpi: three days post injection with *E. ictaluri*. Different letters indicate differences among diets at a given time point ($p< 0.05$). Values are means ± SEM, n = 3.

3.3.2. Plasma natural hemolytic complement activity

The treatment containing Pg0.2 was observed to possess the highest ACH50 level compared to other treatments throughout the sampling time points (Fig. 3). The ACH50 activity was increased significantly in Pa0.2-based diet in W3, then the level did not statistically differ compared to control diet in W6 and 3 dpi. On the other hand, Mix0.2-based diet significantly enhanced ACH50 levels compared to control at the end of the feeding trial and post challenge with *E. ictaluri*.

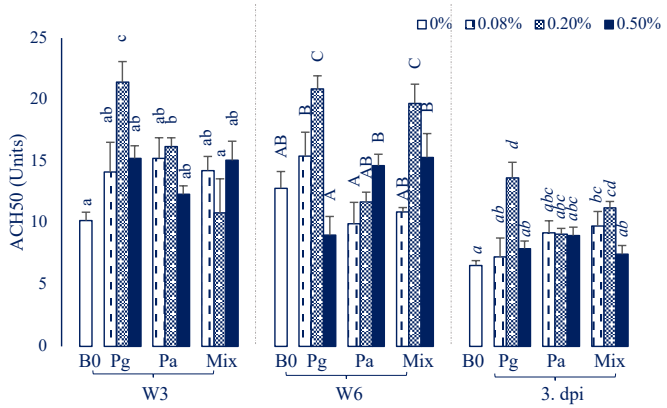

Figure 3. ACH50 activity in plasma of striped catfish fed extract-based diets supplemented with a single supply or a mixture of *P. guajava* and *P. amarus* at 0; 0.08; 0.2 and 0.5% after 3, 6 weeks and 3 dpi. B0: basal diet; Pg: *P. guajava*; Pa: *P. amarus;* Mix: Mixture of P. *guajava* and *P. amarus*; W3: week 3; W6: week 6; 3 dpi: three days post injection with *E. ictaluri*. Different letters indicate differences among diets at a given time point ($p < 0.05$). Values are means ± SEM, n = 3.

3.3.3. Total immunoglobulin

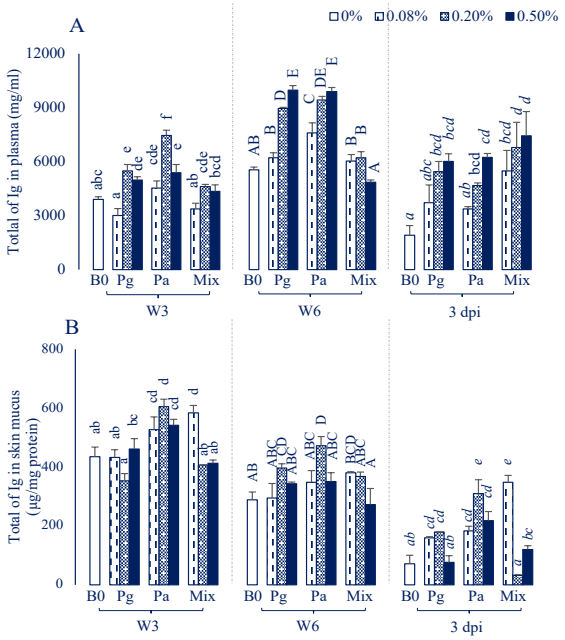

Figure 4. Total of Ig in A) Plasma and B) Skin mucus of striped catfish fed extract-based diets supplemented with a single supply or a mixture of *P. guajava* and *P. amarus* at 0; 0.08; 0.2 and 0.5% after 3, 6 weeks and 3 dpi. B0: basal diet; Pg: *P. guajava*; Pa: *P. amarus;* Mix: Mixture of P. *guajava* and *P. amarus*; W3: week 3; W6: week 6; 3 dpi: three days post injection with *E. ictaluri*. Different letters indicate differences among diets at a given time point ($p < 0.05$). Values are means ± SEM, n = 3.

The statistical analysis showed that single addition of Pg or Pa extracts could strongly induce an increase of the plasma total Ig, whereas dietary administration of extract mixture did not affect the total Ig during the feeding trial (Fig. 4A). Although total Ig decreased after injection

with bacteria, it was significantly higher in some extract treatments than in the control group ($p < 0.05$).

Dietary administration of single or combined Pg and Pa had positive effects on total Ig throughout the experiment (Fig. 4B). In particular, the total Ig notably increased in three concentrations of Pa-based diets in comparison to control in W3 and after the challenge test ($p < 0.05$).

3.4. Disease resistance against *E. ictaluri*

Single or combined Pg and Pa extract-based diets significantly reduced striped catfish mortality after injection with *E. ictaluri* in W6 (Fig. 5). The fish mortality in the control group was 47.62%. Fish fed Pa0.5 and Mix0.5 had the same lowest mortality rate (4.76%) compared to control ($p < 0.05$). Similarly, the mortalities were statistically reduced in Pg0.2, Pg0.5, Pa0.2 and Mix0.08 groups compared to control. No mortalities were observed in the negative control group. *E. ictaluri* were detected in all bacterial infection treatments.

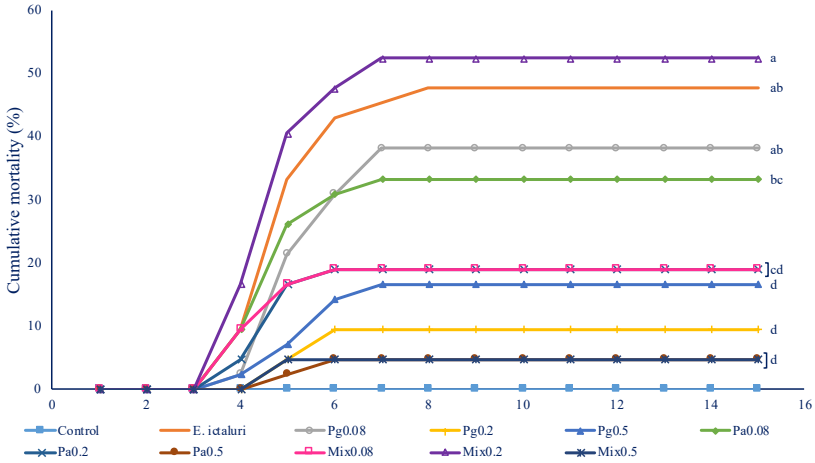

Figure 5. Fish mortality in 15 days after challenge test with *E. ictaluri* at week 6. B0: basal diet; Pg: *P. guajava*; Pa: *P. amarus;* Mix: Mixture of P. *guajava* and *P. amarus*. Different letters indicate differences among diets ($p < 0.05$). Values are means ± SEM, n = 3.

3.5. Effect of extract-based diets on liver proteome profile

3.5.1. Overall hepatic protein profiling

A total of 2484 proteins were identified within the seven experimental groups of striped catfish liver with a false discovery rate of 1%, of which 1794 proteins were quantified (Table Suppl. 1). The GO annotation analysis predicted that these proteins were involved in different functions related to a biological process, cellular component and molecular function (Fig. 6). Among the biological processes, cellular processes (55.9%) and biological regulation (44%) were the major functions of striped catfish liver. Subcategories including cells (60.6%), cell parts (60.5%) and organelles (53.8%) were commonly represented in the cellular components. Regarding molecular function, the binding subcategory was found to be the most frequent at 57.6%.

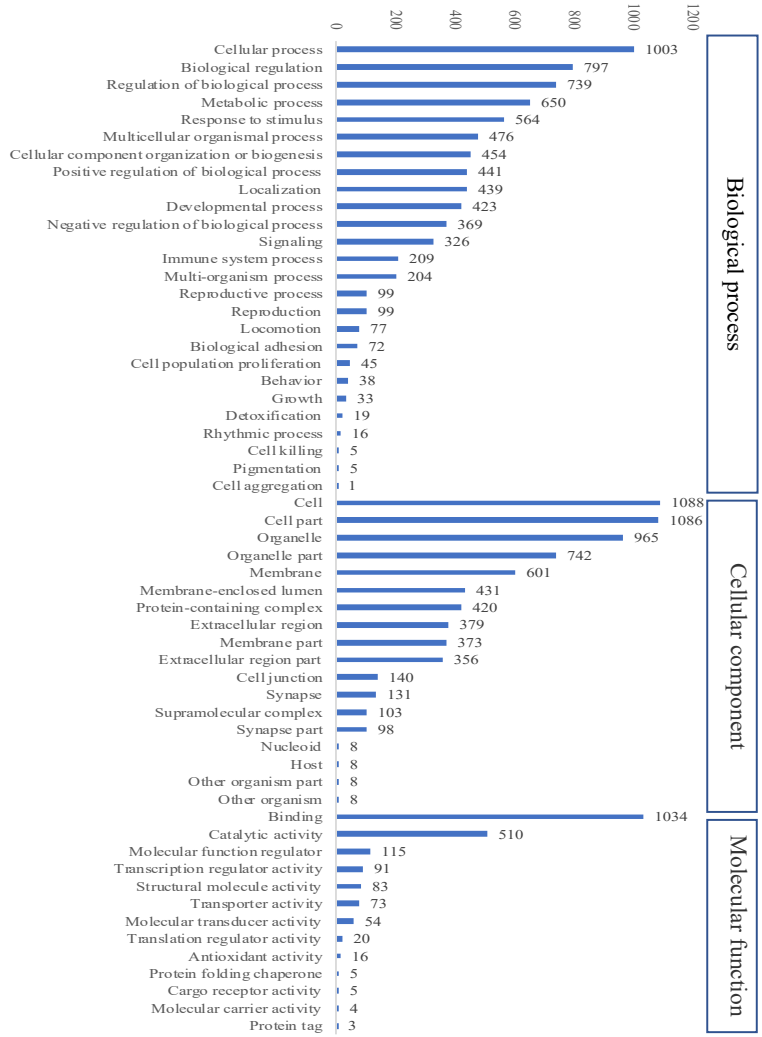

Figure 6. Gene Ontology (GO) analysis of all identified proteins in striped catfish liver after 6 weeks of extract-based diets. The number of proteins in three categories including Cellular component, Biological process and Molecular function.

3.5.2. Comparative DEPs in extract-based diets

The comparison of DEPs in extract-based versus control diets shown using volcano plots indicated that more proteins were downregulated than upregulated, with a fold change above 1.2 (Fig. 7). Mix0.5 versus control group showed the greatest proportion (45.6%) between the number of upregulated and total DEPs compared to other extract-based groups. The lowest proportion of upregulated proteins was 3.9% in Pa0.08-based diet versus control, followed by Pg0.5 versus control (9.9%), Pg0.08 versus control (19%), Mix0.08 versus control (22.2%) and Pa0.5 versus control (22.6%). In contrast, the number of downregulated DEPs in each extract-based diet versus control was the highest in Pa0.08 and the lowest in Mix0.5. Moreover, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NLR family CARD domain-containing protein 3 (NLRC3), caspase-8 (CASP8), zinc finger protein 501 (ZNF501), Erlin-1 (ERLIN1), keratin-type I cytoskeletal 18 (KRT18) and HMG domain-containing protein 4 (HMGXB4) were always represented as the top five upregulated proteins in at least two extract-based treatments.

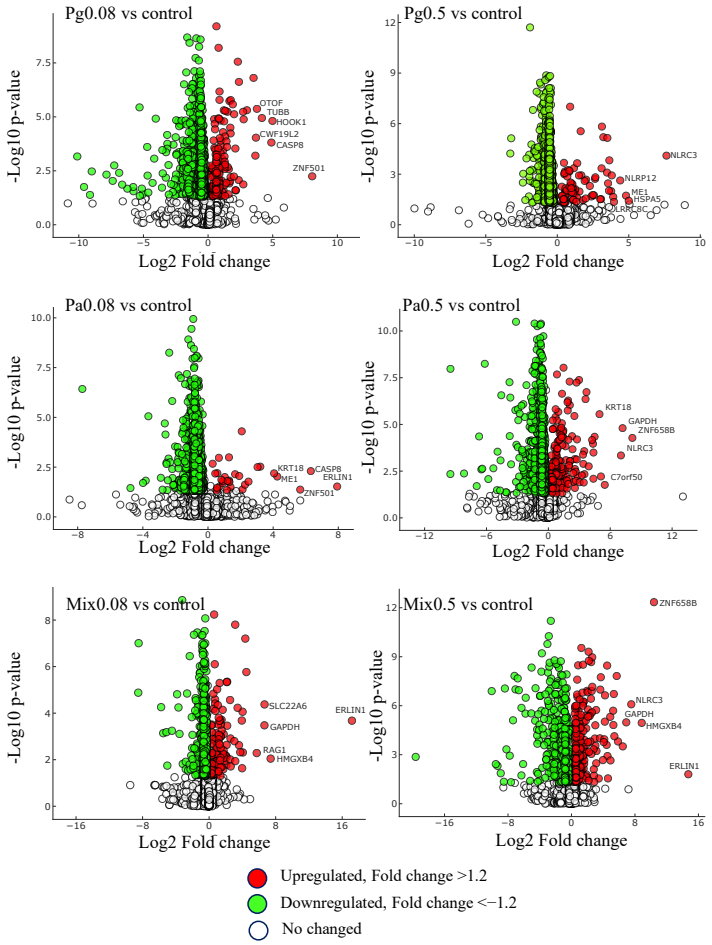

Figure 7. Distribution of up- and down-regulated proteins and their P values. Fold changes are relative to extract-based diets versus control diet. Pg: *P. guajava*; Pa: *P. amarus;* Mix: Mixture of P. *guajava* and *P. amarus*. Proteins satisfying |fold change| ≥ 1.2 and p<0.05. The horizontal and vertical axis represent log_2Fold-change and -log_{10}P-value, respectively.

3.6. Extract-based diets affect the liver proteome network related to antioxidant, immune and stress responses

3.6.1 GO functional enrichment classification of the DEPs

By performing the GO enrichment analysis, 238 DEPs focusing on immune system processes, response to stimulus (innate immune response, response to oxidative stress, inflammatory response and defense response to other organisms), a metabolic process (oxidation-reduction process) and cellular process (apoptotic process) were classified. The results indicated that there were 11 GO clusters belonging to immune system processes, with the most abundant DEPs grouped into immune response (71 proteins), followed by leukocyte activation (57 proteins) and immune effector process (54 proteins), whereas leukocyte migration and myeloid cell homeostasis (10 proteins) were less abundant in this group. Up to 103 proteins were involved in the oxidation-reduction process of metabolism; 63 proteins were classified into the subclasses of response to stimulus; and 40 proteins related to the apoptotic process (Fig. 8A; Table Suppl. 2). The comparison of the different GO annotations indicates that there was a

strong overlap between the GO functions (Fig. 8B). Two DEPs including PRDX3 (peroxiredoxin-3) and GAPDH were represented in all the four GO enriched pathways.

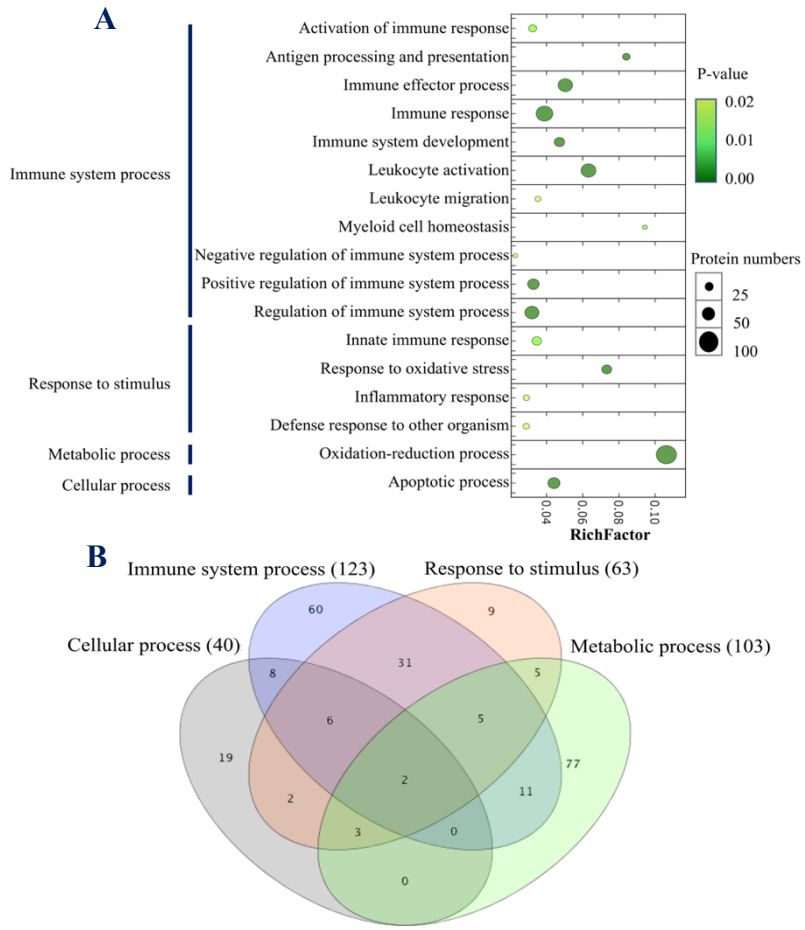

Figure 8. Scatter plot of GO enrichment analyses (A) and hierarchical clustering (B) of differentially abundant proteins related to immune system process. The Y-axis is different GO functions. The X-axis is the value of rich factor (p-value of Fisher exact test <0.05). The color of circles stands for the p-value of function. The size of circles stands for protein numbers.

Regarding the functional categories mentioned above, 238 DEPs were measured across the groups of extract-based diets with the control treatment (Fig. 9; Table Suppl. 3). Mix0.5 group induced an upregulation in all GO categories, the ratios between upregulated versus downregulated DEPs being 52 versus 19 (immune system process), 24 versus 15 (response to stimulus), 17 versus 6 (apoptotic process) and 36 versus 33 (oxidation-reduction process). GO enrichment analysis also revealed that apoptotic process and response to stimulus categories were upregulated in Pa0.5-based diet, while the Mix0.08 group could induce the upregulation of apoptotic process. Enriched proteins were mostly downregulated in all tested categories in Pg0.08, Pg0.5 and Pa0.08 groups. Among the GO enriched categories, six proteins coding for the NLR family CARD domain-containing protein 3 (NLRC3), tubulin beta chain (TUBB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NACHT, LRR and PYD domains-containing protein 12 (NLRP12), recombination activating protein 1 (RAG1) and caspase-8 (CASP8) were highly upregulated in immune system processes. In response to stimulus,

proteins namely CASP8, GAPDH and keratin (KRT18) were highly upregulated. The expression of two proteins encoding GAPDH and TUBB were highly increased in the apoptotic process. Only the GAPDH protein was highly expressed in the oxidation-reduction process.

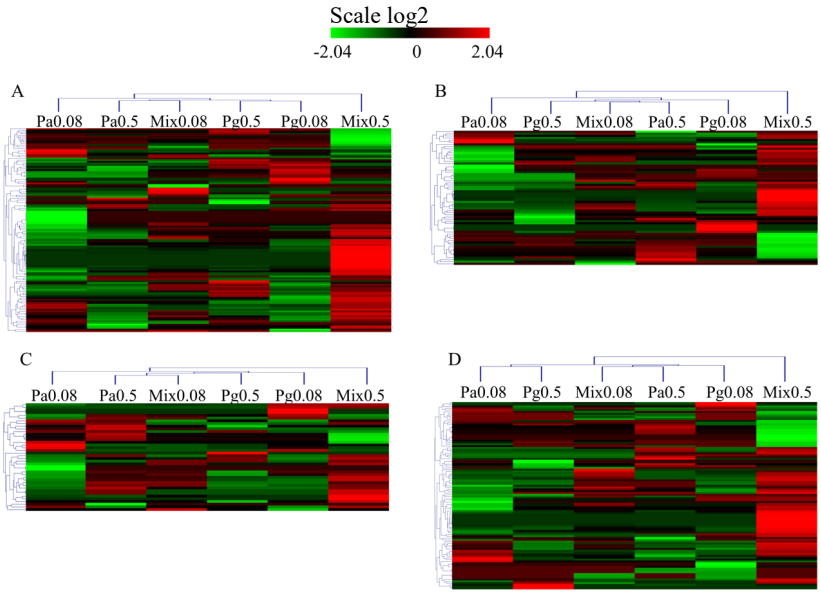

Figure 9. Heatmap of GO and protein domain enrichment analysis of DEPs related to A) Immune system process, B) Response to stimulus (response to stress), C) Apoptotic process and D) Oxidation-reduction process in extract-based diets compared to control diet. Heatmap represents differentially expressed proteins in extract-enriched diets (|fold change| > 1.2, p <0.05).

3.6.2. Differentially regulated KEGG pathways related to antioxidant and immune response among extract-based diets

All the DEPs were mapped in the KEGG database to search for the proteins involved in significant immune and antioxidant-related pathways. The results indicated that in total seven immune and antioxidant related KEGG pathways were significantly enriched in most extract-based treatments ($p < 0.05$) (Fig. 10). In comparison to the control diet, Mix0.5-based diet significantly upregulated the expression of proteins that were involved in antigen processing and presentation, apoptosis, leukocyte transendothelial migration, natural killer cell mediated cytotoxicity, and some signalling pathways such as calcium and NOD-like receptor. Moreover, upregulated proteins involved in pathways related to apoptosis, leukocyte transendothelial migration, natural killer cell mediated cytotoxicity and the calcium signalling pathway were found in Mix0.08. Regarding Pa extract-based diets, Pa0.5 induced the upregulation of proteins involved in some KEGG pathways including apoptosis, leukocyte transendothelial migration, glutathione metabolism and oxidative phosphorylation. In parallel, most of the proteins related to the calcium signalling pathway and leukocyte transendothelial migration decreased in expression following Pa0.08-based diet. On the other hand, in Pg0.5-based diet leukocyte transendothelial migration related proteins were upregulated, whereas proteins related to calcium signalling pathway were downregulated. Upregulated proteins involved in leukocyte

transendothelial migration as well as downregulated proteins in antigen processing and presentation, calcium and NOD-like receptor signalling pathways were also enriched in Pg0.08 group.

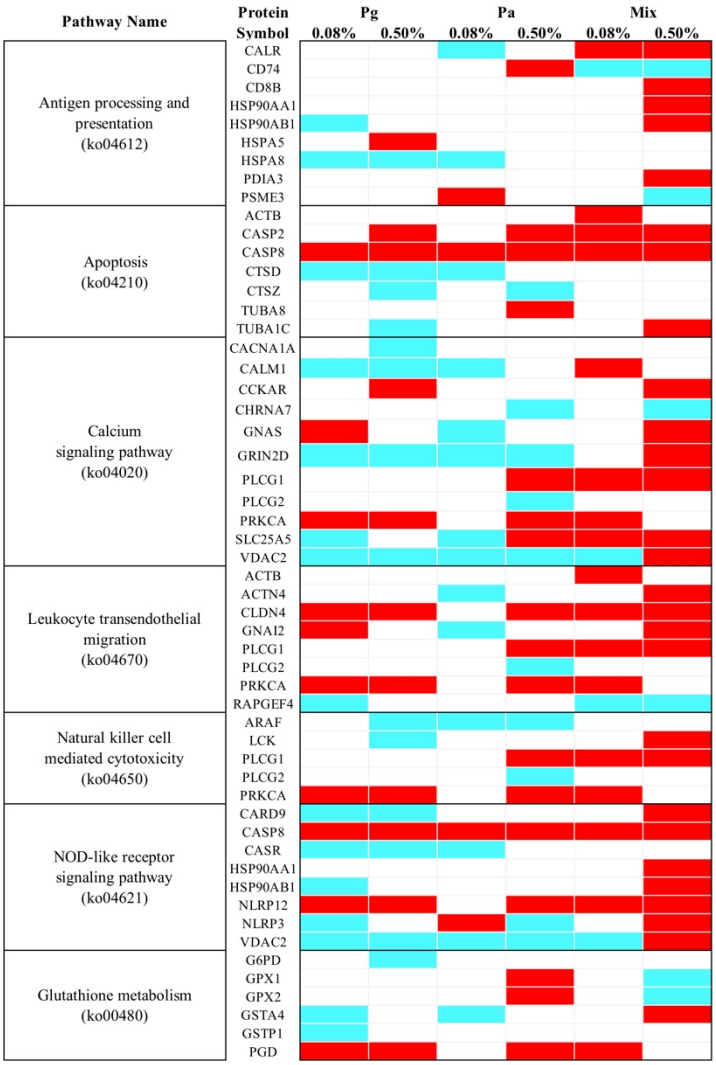

Figure 10. Heatmap of KEGG pathway enrichment analysis for immune and antioxidant related DEPs. Cyan: significant downregulation, Red: significant upregulation, White: no change

Among the nine proteins predicted to be related to antigen processing and presentation, T-cell surface glycoprotein CD8 beta chain (CD8B), heat shock protein HSP 90-alpha 1 (HSP90AA1), HSP 90-beta (HSP90AB1) and protein disulfide-isomerase A3 *(*PDIA3) additively increased in the Mix0.5 group compared to other experimental groups. caspase 8 (CASP8), predicted to belong to both NOD-like receptor signalling and apoptosis pathways, significantly increased in all extract treatments. Several proteins such as tubulin alpha-1C chain (TUBA1C), cholecystokinin receptor type A (CCKAR), guanine nucleotide-binding protein G(s) subunit alpha (GNAS), glutamate receptor ionotropic NMDA 2D (GRIN2D), PLCG1 (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1), PRKCA (protein kinase

C alpha type), ADP/ATP translocase 2 (SLC25A5), voltage-dependent anion-selective channel protein 2 (VDAC2), alpha-actinin-4 (ACTN4), guanine nucleotide-binding protein G(i) subunit alpha-2 (GNAI2), tyrosine-protein kinase Lck (LCK), caspase recruitment domain-containing protein 9 (CARD9), NACHT, LRR and PYD domains-containing protein 12 (NLRP12) and NLRP3 were differentially expressed depending on the dose of extracts. Furthermore, most DEPs involving glutathione metabolism (i.e., glutathione peroxidase 1 (GPx1), glutathione peroxidase 2 (GPx2), 6-phosphogluconate dehydrogenase (decarboxylating) (GPD)) were upregulated in Pa0.5 group only.

3.6.3. DEPs involved in lipid metabolism

Dietary supplementation with Pg and Pa extracts significantly altered protein expression levels related to lipid metabolism in plant extract-based groups compared to the control group. KEGG pathway enrichment analysis showed that a total of 15 DEPs were reported to be distributed into four significant subcategories involved in lipid metabolism such as fatty acid degradation (fatty acid metabolism, glycerolipid metabolism and sphingolipid signalling pathways (Table 3)). Most proteins were significantly downregulated in at least one extract group compared to control group. However, only protein kinase C alpha type (PRKCA) belonging to the sphingolipid signalling pathway was highly upregulated in four groups including Pg0.08, Pg0.5, Pa0.5 and Mix0.08; guanine nucleotide-binding protein G(i) subunit alpha-2 (GNAI2) of the sphingolipid signalling pathway and 4-trimethylaminobutyraldehyde dehydrogenase (ALDH9A1) related to glycerolipid metabolism were upregulated in Mix0.5 group only; long-chain specific acyl-CoA dehydrogenase (mitochondrial ACADL) related to fatty acid degradation and fatty acid metabolism significantly increased in the Pa0.5 group; alcohol dehydrogenase 5 class-3 (ADH5) related to fatty acid degradation considerably increased in expression in the Pa0.08 group.

Table 3. List of differentially express proteins in striped catfish liver related to lipid metabolism after dietary administration plant extracts

Description	Accession	Protein Name	Pg		Pa		Mix		Mass ($\times 10^3$)
			0.08	0.5	0.08	0.5	0.08	0.5	
Fatty acid degradation (KEGG:ko00071)									
Long-chain specific acyl-CoA dehydrogenase- mitochondrial	AHH41666.1	ACADL				1.3			49.4
Peroxisomal acyl-coenzyme A oxidase 1	XP_017337623.1	ACOX1	-1.2						74.7
Peroxisomal acyl-coenzyme A oxidase 3	XP_017327584.1	ACOX3						-2.2	78.9
Alcohol dehydrogenase class-3	XP_017548877.1	ADH5	-1.6		2.0			-1.3	41.8
Aldehyde dehydrogenase- mitochondrial	XP_017307563.1	ALDH2	-1.6	-1.6	-1.3	-1.9	-1.5	-1.4	57.4
Fatty aldehyde dehydrogenase	XP_022531853.1	ALDH3A2	-1.7			-1.6			55.1
Alpha-aminoadipic semialdehyde dehydrogenase	XP_007252494.1	ALDH7A1	-2.0	-1.9		-2.1	-1.4	-1.4	59.3
4-trimethylaminobutyraldehyde dehydrogenase	XP_007250712.2	ALDH9A1			-1.9			1.2	57.0
Trifunctional enzyme subunit beta- mitochondrial	XP_007260524.2	HADHB	-1.6	-1.6	-1.4	-1.7	-1.6	-1.3	50.6
Fatty acid metabolism (KEGG:ko01212)									
Long-chain specific acyl-CoA dehydrogenase- mitochondrial	AHH41666.1	ACADL				1.3			49.4
Peroxisomal acyl-coenzyme A oxidase 1	XP_017337623.1	ACOX1	-1.2				-1.2		74.7
Peroxisomal acyl-coenzyme A oxidase 3	XP_017327584.1	ACOX3						-2.2	78.9

Trifunctional enzyme subunit beta-mitochondrial	XP_007260524.2	HADHB	-1.6	-1.6	-1.4	-1.7	-1.6	-1.3	50.6
Glycerolipid metabolism (KEGG:ko00561)									
1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	AHH40462.1	AGPAT5			-1.9				42.4
Aldose reductase	XP_017321050.1	AKR1B1	-1.7	-2.0	-1.4	-1.8	-1.3	-1.5	36.8
Aldehyde dehydrogenase mitochondrial	XP_017307563.1	ALDH2	-1.6	-1.6	-1.3	-1.9	-1.5	-1.4	57.4
Fatty aldehyde dehydrogenase	XP_022531853.1	ALDH3A2	-1.7			-1.6			55.1
Alpha-aminoadipic semialdehyde dehydrogenase	XP_007252494.1	ALDH7A1	-2.0	-1.9		-2.1	-1.4	-1.4	59.3
4-trimethylaminobutyraldehyde dehydrogenase	XP_007250712.2	ALDH9A1			-1.9			1.2	57.0
Sphingolipid signaling pathway (KEGG:ko04071)									
Cathepsin D	XP_007250635.1	CTSD	-1.2	-1.5	-1.3				43.6
Guanine nucleotide-binding protein subunit alpha-13	XP_007259155.2	GNA13						-33.6	44.3
Guanine nucleotide-binding protein G(i) subunit alpha-2	XP_022531259.1	GNAI2			-1.7			1.4	40.9
Protein kinase C alpha type	XP_017314924.1	PRKCA	4.3	2.0		3.2	2.6		79.5

4. Discussion

Manipulation of health status using plant extracts has been developed in fish as an ecological practice for sustainable aquaculture in order to improve growth performance, enhance the immune response and to control mortality due to environmental pathogens (5, 36). From a large *in vitro* screening (using striped catfish peripheral blood mononuclear cells and head kidney leukocytes) of extracts from 20 plants commonly used in traditional fish prophylaxis in Vietnamese aquaculture (25) followed by *in vivo* validation (26), we previously found that five ethanol plant extracts including *P. amarus*, *P. guajava*, sensitive plant *Mimosa pudica* L., neem *Azadirachta indica* A. Juss and asthma plant *Euphorbia hirta* L. were potentially effective in modulating blood parameters, immune responses and providing better protection of striped catfish against bacterial infection with *E. ictaluri* after 8 weeks of feeding. Pg and Pa also displayed antioxidant and immunomodulatory properties due to the presence of various biological compounds (8, 10, 37-39). In the present study, the immunomodulatory effects of Pg and Pa extracts alone or in combination were assessed by evaluating their capacities to improve immune parameters and resistance to *E. ictaluri* infection. Notably, this study also provided an insight into the changes observed in the immune proteome of striped catfish liver after feeding with extract-based diets.

4.1. Growth performance and feed utilisation

After 6-weeks feeding, the highest values of WG and SGR were observed in Pg0.2 groups, confirming that Pg supplemented at its optimal dose could improve the growth of striped catfish. FCR values lower than control were observed in most of the plant extract-based treatments, but the significantly lower FCR values were only achieved in the groups fed Pg0.2 and Pa0.2 diets. The lower FCR value in fish fed plant extract-based diets suggests an improvement in feed utilisation for fish growth, thus reducing the cost of production. However, the enhancement of growth performance by extract-based diets may occur in a species, time and concentration-dependent manner. Giri et al. (11) indicated that guava leaf-enriched diets at 0.5% significantly increased the growth performances of rohu in 60 days. The growth and the nutritional indicators of Nile tilapia *Oreochromis niloticus* fingerlings were considerably improved after 84 days of feeding with 0.5%, 0.75% and 1% ethanol guava extracts (40). Another study by Gobi et al. (13) reported that a considerable increase of final weight and SGR of *O. mossambicus* as well as a decrease of FCR were observed with a 1% ethanol guava leaf extract-based diet in a shorter time of 30 days of feeding.

4.2. Immune response

4.2.1. Cellular immune response

Phagocytes constitute the primary limitation of invasive pathogens by releasing ROS and NOS (41, 42). Our study highlighted that the spleen RBA significantly increased in only Pa0.2-based diet compared to control in W3, whereas the NOS level was found to significantly decrease in Pg0.2-based diet at the same time. The results also could not detect significant differences in RBA and NOS activities in all extract-based diets compared to control in W6. In line with our

results, Yin et al. (43) also showed that Nile tilapia fed diets supplemented with *Astragalus radix* and *Scutellaria radix* extracts did not significantly increase ROS production, even though lysozyme activity was enhanced. Induced NOS synthase (iNOS) in response to immunostimulatory signals is involved in NOS production (44). Giri et al. (11) indicated that rohu fed guava extracts for 60 days had significantly lower *inos* transcript expression in head kidney, intestine and hepatopancreas at dose of 1 and 1.5%. Moreover, significant dose-dependent inhibitions of NOS production were observed in male Balb/c mice after 14 days of Pa administered by oral gavage (10). In contrast, tilapia fed diets supplemented with 0.1, 0.5 and 1.0% ethanol guava extract displayed significantly higher RBA and NOS levels at day 30 ($p < 0.05$) (13). Similarly, the leaf of *P. guajava* and mango *Mangifera indica* extract-based diets also significantly enhanced RBA production in rohu after a 35-day feeding period (12). As mentioned above, ROS are generated by neutrophils and macrophages during bacterial infection, while NOS is created as a signalling molecule in ROS generation during phagocytosis (45). Enhanced activities of ROS and NOS are seen as indicators of immune condition. Our study also found that the RBA and NOS levels were highly increased in all treatments after *E. ictaluri* infection. Notably, the RBA levels were statistically enhanced in Pa0.08, Pa0.2 and Mix (all dose) treatments compared to control, whereas only the Pg0.2-enriched diet could stimulate the increase of NOS after the challenge test ($p < 0.05$). The reactive nitrogen and oxygen species could have possible beneficial effects at lower levels (46). The positive and negative aspects of ROS should be carefully balanced. The fish's proteins, lipids or DNA will be damaged if ROS levels become too high (47).

4.2.2. Humoral immune response

In aquatic animals, the humoral innate immune system has been considered as a vital weapon in protecting fish against opportunistic pathogens (48). Lysozyme acts as an important indicator of the non-specific humoral immune response. In addition, lysozyme possesses both bactericidal and opsonin effects that result in activation of the complement system and phagocytes to prevent infectious diseases (49). Besides the non-specific humoral immune system, total immunoglobulin also plays an essential role in host defense mechanisms and acts as a biomarker for fish adaptive immunity (50). The current study revealed that the extract-based diets considerably increased serum lysozyme, complement activities, as well as total immunoglobulin levels throughout the experiment in a concentration and time-dependent manner. In agreement with our results, several studies also indicated that Pg or Pa extract-based diets positively enhanced lysozyme, complement activities and total Ig in rohu (11, 12), tilapia (13), common carp (14) and striped catfish (26). After infection by *E. ictaluri*, the lysozyme and complement activities as well as the total Ig mostly decreased but were still significantly higher in some extract-based diets compared to those of the control. However, the lysozyme activity was significantly reduced in mixture-based diets compared to control. These results are consistent with our previous study showing that the humoral immune parameters (i.e., lysozyme, complement activities and total Ig) in Pg and Pa-based diets were significantly higher than those of the control diet after a bacterial challenge test (26).

Skin is a major line of entry for infectious pathogens in aquatic animals. Therefore, skin mucus contains various biologically active components (including defensive molecules) (51). In the present study, single or combined administrations of Pg and Pa increased lysozyme activity in skin mucus throughout the experiment, although the increases were dependent on concentration and time of sampling. Total immunoglobulin was also significantly elevated in skin mucus of striped catfish following the administration of all plant extract-based diets compared to control. In agreement with these results, we previously also found that Pg and Pa extract-based diets at 0.2 and 1.0% considerably enhanced lysozyme activity as well as total Ig in skin mucus after an 8-week feeding period (26). A study by Azimirad et al. (52) reported that single or combined administration of fructooligosaccharide and *Pediococcus acidilactici* increased immune markers (i.e., lysozyme activity, total immunoglobulin and protease activity) in skin mucus of angelfish *Pterophyllum scalare*. In an 8-week feeding trial, the total Ig was also considerably increased in common carp fed 1.0% Pg leaf extract (14).

The elevation of cellular immune response in association with humoral immune response in serum and skin mucus contributes to enhancing the defense mechanism by destroying invading pathogens. Our findings also revealed that single or combined Pg and Pa extract-based diets had a positive influence on the survival of striped catfish after *E. ictaluri* infection, especially in Pg0.2, Pg0.5, Pa0.2, Pa0.5, Mix0.08 and Mix0.5 groups. Improving the survival rates in striped catfish against *E. ictaluri* infection demonstrated in this study were in agreement with the previous reports that indicated Pg extract-based diets significantly reduced mortalities in rohu (11, 12), tilapia (13) and Nile tilapia (40) after challenge with *A. hydrophila*. In the current study, the combination of Pg and Pa extracts had a positive impact on the disease resistance of striped catfish.

4.3. Liver proteome profile

The liver is one of the most important organs that participates in metabolism and nutrient storage, as well as the immune response (20). In this study, the comparison of the liver proteome of striped catfish fed diets enriched by single or combined Pg or/and Pa extracts was investigated. A total of 2484 proteins have been reported by LC-IMS-QTOF-MS, from which 1794 proteins were quantified in in striped catfish liver. The current findings revealed that cellular processes and biological regulation were the major biological processes affected in striped catfish liver. The top three subcategories including cells, cell parts and organelles were majority in cellular components. Regarding molecular functions, the subcategory of bindings was found to be most abundant. Similar contributions of the subcategories were also suggested by Wen et al. (22), although the metabolic process was found to be the second major biological process in obscure pufferfish liver. However, cellular processes and metabolic processes were the top two subcategories of biological processes in mice liver treated with sucralose (53). Our results also indicated that a number of proteins were differentially expressed according to the dose and species of plant extracts added to striped catfish diets. When compared to control, Mix0.5 showed the highest proportion of upregulated proteins and, more globally, total DEPs. These proportions were reduced in Pa0.5, Mix0.08, Pg0.08, Pg0.5 and Pa0.08. This outcome

suggests that both single and combined Pg and Pa extract-based diets directly changed the expression of proteins in striped catfish liver.

4.3.1. Effect of extract-based diets on regulating immune system functions and antioxidant metabolism

To illustrate the functions of DEPs among the extract treatments, GO and KEGG analyses were initially used with up and downregulated DEPs. By comparing the protein expression profiles between six plant extract-based diets and the control diet, 17 subcategories involving immune system processes, response to stimulus, the oxidation-reduction process, as well as the apoptotic process were significantly enriched. Moreover, proteins found to be involved subcategories examined above had many regulatory overlaps. Aside from the GO terms, 7 KEGG pathways were significantly co-enriched with proteins related to immune and antioxidant responses. Our findings also showed that the expression levels of proteins related to the tested pathways were differentially regulated in both an extract-type and concentration-dependent manner.

After 6 weeks of feeding with diets supplemented with plant extracts, several immune pathways were significantly enriched in striped catfish liver, such as antigen processing and presentation, leukocyte transendothelial migration, natural killer cell mediated cytotoxicity, the calcium signalling pathway, the NOD-like receptor signalling pathway and apoptosis.

It was reported that antigen processing and presentation play a key role in adaptive immunity in teleost fish (54, 55), which is essential for T cell immune response triggering. The antigen processing and presentation pathway was also enriched in beef cattle fed a mixture containing dry corn grain, corn silage, soybean, citrus pulp pellets, urea, calcareous, mineral salt and potassium chloride (56). Our results showed that most of the proteins involved in antigen processing and presentation were significantly upregulated in Mix-based diets (Mix0.08 and Mix0.5), while those protein expressions were inhibited in Pg0.08. Among nine proteins belonging to antigen processing and presentation, CD8B was significantly upregulated in Mix0.5 group, although the expression level of CD8B was not changed in single diets supplemented only by Pg or Pa. $CD4^+$ and $CD8^+$ are vital in modulating the immune response in the regulation of cytokines (57). In Wistar-Kyoto rats, Pa possessed effective immunosuppressive activities in the cellular immune response. There was a significant decrease in the expression of *cd4*+ and *cd*8+ genes in spleen and serum of rats after treatment with Pa in the presence of lipopolysaccharide (58). Phyllanthin isolated in Pa also caused a significant reduction in the percentage expression of *cd4*+ and *cd*8+ genes when supplemented in Balb/C mice diets (59). In the present study, HSP90AA1 and HSP90AB1 were predicted to be involved in both antigen processing and presentation and NOD-like receptor signalling pathways. Moreover, both HSP90AA1 and HSP90AB1 expressions were significantly upregulated in the Mix0.5 group, whereas HSP90AB1 level was reduced in the Pg0.08 group only. Upregulation of *hsp90* following curcumin-based diets in pool barb *Puntius sophore* has also been reported by Mahanty et al. (60). A previous study suggested that in loach *Misgurnus anguillicaudatus hsp70*, *hsp90* alpha and *hsp90* beta, which are associated more specifically

with thermal stress, were strongly upregulated after the fish were fed vitamin C (61). Cold stress-low temperature was proven to induce HSP90 transcriptional expression in pufferfish liver (62). PDIA3 is mainly present in the endoplasmic reticulum and is crucially involved in the folding process of MHC class I (63). CALR-calreticulin is also important in MHC class I antigen processing and presentation. The upregulation in expression of CD8B, PDIA3, CALR as well as HSP90AA1 and HSP90AB1 in the liver could be associated with the activation of the antigen processing and presentation pathway in the Mix0.5 group.

The leukocyte transendothelial migration pathway has also been associated with adhesion molecules, chemokines and cytoskeletal regulators (64). In response to inflammatory signals, leukocytes leave the bloodstream by crossing the endothelial monolayer which results in changes to the adhesive properties and shape of cells (64). Moreover, several molecules in the leukocyte transendothelial migration pathway were also activated in response to immune challenge at different metamorphosis stages in the grouper (*Epinephelus coioides*) (65). In particular, several proteins present in the leukocyte transendothelial migration pathway were significantly upregulated in striped catfish fed Pg0.08, Pg0.5, Pa0.5, Mix0.08 and Mix0.5.

Moreover, natural killer cell mediated cytotoxicity is essential for the first line immune defense against invading pathogens as well as for modulation of liver injury (66). Several proteins involved in natural killer cell mediated cytotoxicity were also noticeably upregulated in Mix groups. Calcium signalling plays a critical role in many cellular processes including activation of inflammasomes (67). Increasing intracellular calcium could damage the mitochondria and then activate the NLRP3 inflammasome through the release of ROS (68). The present results also revealed increased expression of three proteins (PRKCA, PLCG-1 and 2) related to natural killer cell mediated cytotoxicity, the calcium signalling pathway and leukocyte transendothelial migration, suggesting a link between these immune subcategories.

The NOD-like receptor signalling pathway contributes to many crucial activities including autophagy, apoptosis and development (69). In humans, NOD-like receptors act as major players in the interface between innate immunity and cancer. The NOD-like receptor family of proteins is vital in mediating the initial innate immune response, as well as having roles in cellular injury and stress (70). Of the 8 proteins involved in the NOD-like receptor signalling pathway, seven proteins were highly upregulated with the Mix0.5-based diet. NLRP3 is an intracellular sensor whose activation leads to the formation of an inflammasome in leukocytes (70). Specifically, NLRP3 positively regulates apoptosis through the activation of CASP8 (71). Similarly, NLRP12 acts as a proinflammatory protein in caspase-1 signalling (72). NLRP12 was upregulated in most of the plant extract groups, except in Pa0.08, whereas extract-based diets dysregulated the expression of NLRP3 in a dose-dependent manner.

The present study also found that many proteins involved in apoptosis were significantly upregulated in Pa0.5, Mix0.08 and Mix0.5 groups. Apoptosis is a physiological programme that is a type of programmed cell death and plays a key role in the regulation and functioning of the immune system (73). In liver, apoptosis is a physiological process to eliminate damaged or infected cells and maintain tissue homeostasis (74). The apoptosis process is mediated by a

family of aspartate-specific cysteine proteases known as caspases (75). Notably, our results revealed the level of CASP8 considerably increased in all extract-based diets compared to control, which was predicted to be involved in both the NOD-like receptor signalling pathway and apoptosis. In parallel, the expression of CASP2 was statistically increased in Pg0.5, Pa0.5, Mix0.08 and Mix0.5. In line with our results, Van et al. (76) also demonstrated that guava leaf extract increased the expression of proteins involved in apoptotic processes including caspases 3, 8 and 9 in HepG2 cells. The same results were reported by Mbaveng et al. (2016), indicating that Pg bark extract induced apoptosis in leukemia CCRF-CEM cells via caspase (caspase 3/7, 8 and 9) activation (77). Similarly, Pa induced apoptosis in Dalton's lymphoma ascite cells in mice through activation of *casp3* mRNA (78). These effects might be due to the presence of phenolic compounds in the extract (79, 80). The activation of caspases results in DNA fragmentation, destruction of the nuclear proteins and cytoskeleton, crosslinking of proteins, expression of ligands for phagocytic cells and formation of apoptotic bodies (81, 82). It should be noted that the combination of Pg and Pa extracts resulted in the upregulation of higher levels of proteins related to antigen processing and presentation, natural killer cell mediated cytotoxicity, the calcium signalling pathway and the NOD-like receptor signalling pathway, suggesting possible synergistic effects of these bioactive compounds when compared to plant extracts alone.

Furthermore, the proteomic analysis in liver also revealed different regulations of glutathione metabolism among experimental groups compared to the control group. Notably, six proteins were directly regulated and functionally related to antioxidation. The results suggested that Pa0.5-based diet had significant effects on the regulation of antioxidation in striped catfish liver. It has been shown that the presence of phenolic compounds in plant extracts greatly contributes to their antioxidant potential (83, 84). Furthermore, Pa contains a rich source of hydrolyzable phenols including phyllanthin and hypophyllanthin (85) which are highly responsible for antioxidant activity (86). Among the proteins, glutathione peroxidase is important in preventing many molecules from being damaged by oxidative effects during oxidative stress (87, 88). Antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were also increased in tilapia fed guava leaf extract (13), whereas Pa significantly stimulated the increase of GPx activity in rat plasma (89). In our study, glutathione peroxidase 1 (GPx1) and glutathione peroxidase 2 (GPx2) expression was upregulated in the Pa0.5 group, while both proteins were downregulated in the Mix0.5 group. These findings suggest that the combination of Pa and Pg did not support any possible synergistic effect on antioxidant activity.

4.3.2. Effect of extract-based diets on lipid metabolism

Single or combination Pg and Pa extract-based diets significantly reduced protein abundances related to lipid metabolism (i.e., fatty acid degradation, fatty acid metabolism, glycerolipid metabolism and sphingolipid signalling pathways), suggesting a negative effect of the extracts on the lipid metabolism process in striped catfish liver. This could explain why growth did not significantly increase in 0.08 and 0.5% extract-based diets in W6. Lipid metabolism is a complex process and the key proteins related to this process play important roles in animals

(90). Our results showed that only PRKCA, which belongs to the sphingolipid signalling pathway, was considerably upregulated in Pg0.08, Pg0.5, Pa0.5 and Mix0.08 groups. Long-chain specific acyl-CoA dehydrogenase (ACADL) is thought to be involved in both fatty acid degradation and fatty acid metabolism and was upregulated in Pa0.5 group only. The decrease of protein levels related to fatty acid synthase was observed in rainbow trout liver following handling stress (91). However, dietary fish oil mixture (EPA/DHA 1:1) consumed together by rats could upregulate fatty acid beta oxidation (ACOX2) after 24 weeks of feeding (21).

5. Conclusion

In conclusion, our findings revealed how single and combined Pg and Pa extract-based diets acted in the regulation of immune responses, significantly reducing fish mortality. They also provide a better understanding about the effects of these plant extracts, alone or in combination, on the liver proteome of striped catfish. The results of humoral and cellular immune responses throughout the feeding experiment did not support additive effects of the plant extract mixture compared to the single extracts. However, the proteomic results showed that the administration of combined *Psidium guajava* and *Phyllanthus amarus* extracts could modulate proteins involved in antigen processing and presentation, leukocyte transendothelial migration, natural killer cell mediated cytotoxicity, the calcium signalling pathway, the NOD-like receptor signalling pathway and apoptosis in striped catfish, whereas single Pg or Pa-enriched diets stimulated the upregulation of fewer pathways (Fig. 11). Only the diet supplemented with 0.5% *P. amarus* could enhance the upregulation of glutathione metabolism. In addition, all extract treatments induced the downregulation of pathways related to lipid metabolism. Moreover, the proteomics data also showed that diets supplemented with mixture of *P. guajava* and *P. amarus* resulted in the additive increased expression of several proteins such as CD8B, HSP90AA1, HSP90AB1, PDIA3, CASP2, CASP8, TUBA1C, CCKAR, GNAS, GRIN2D, PLCG1, PRKCA, SLC25A5, VDAC2, ACTN4, GNAI2, LCK, CARD9, NLRP12 and NLRP3. This outcome suggests a potential synergistic effect of combining both plant extracts for the regulation of striped catfish immune responses, but not on antioxidant activity. The main constituents of the extracts should be identified and quantified, as this can influence the modulation of immune capacity and health in fish.

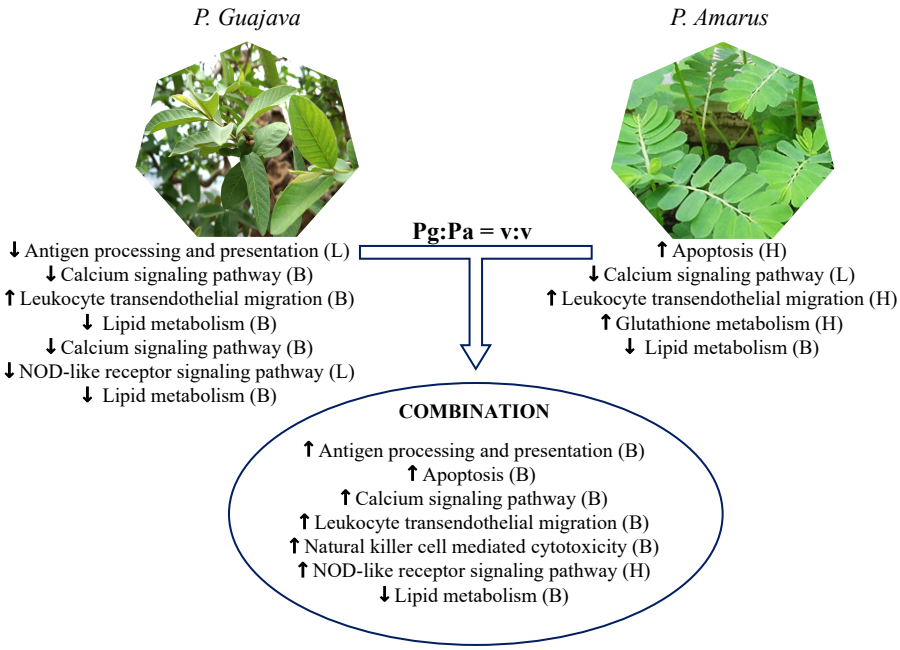

Figure 11. Metabolic pathways in striped catfish liver altered by single versus combination Pg and Pa extract-enriched diets as compared to control diet. L: Low dose examined (0.08%), H: high dose examined (0.5%) and B: both high and low doses.

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Chapter 7

Effects of crude ethanol extracts, their fractions and pure compounds from *Phyllanthus amarus* and *Psidium guajava* on immune responses of striped catfish (*Pagasianodon hypophthalmus*) head kidney leukocytes

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Hypothesis outlines

The previous studies suggested ethanol extracts of *P. guajava* and *P. amarus* are promising for activating the immune system as well as antioxidant status (Chapter 4, Chapter 5, Chapter 6). Extraction is the main process by which bioactive compounds may be obtained from biomass materials. Moreover, several publications indicated that the bioactive constituents depended on the solvent used during extraction. The different polarity of extract solvents could cause a wide variation in the level of bioactive compounds in the extract. The extraction efficiency commonly favors the highly polar solvents, the higher extraction yield was observed in methanol extract, distilled water extract, and ethanol extract compared to chloroform, dichloromethane, and acetone extracts. In the present study, the phytochemical constituents of *P. guajava* and *P. amarus* crude ethanol extracts and their fractions will be evaluated via an HPLC-DAD-Orabitrap-MS analysis, which will be performed by Dr. Nguyen Phuc Dam and PhD student Le Thi Bach. Moreover, in order to better understand the effect of the solvents on biological activities, the present study is trying to investigate the immunoregulatory effects of crude ethanol extracts of *P. amarus* and *P. guajava* as well as their different extract fractions of striped catfish leukocytes. The effects of crude extracts and extract fractions will be measured in the head kidney leukocytes, using three different immunological biomarkers including lysozyme, respiratory burst and nitric oxide species productions. These tested will show how the cells differentially respond to the extracts. Further, our present tests will also how the pure compounds isolated from *P. amarus* and *P. guajava* extracts regulate the immune responses in HKLs, using the same biomarkers.

Abstract

This study aimed to evaluate the immunomodulatory effects of crude ethanol extracts and their fractions (hexane, ethyl acetate, aqueous and non-tannins fractions of *Phyllanthus amarus* Schun and Thonn (Pa) and hexane, dichloromethane, ethyl acetate and aqueous fractions of *Psidium guajava* L. (Pg)) on striped catfish (*Pangasianodon hypophthalmus*) head kidney leukocytes (HKLs). Moreover, several pure compounds identified in crude Pa and Pg extracts

including corosolic acid (Cor), guajaverin (Gua), ursolic acid (Urs), hypophyllanthin (Hyp), avicularin (Avi) and oleanolic acid (Ole) were also investigated for their effects on those cells. HKLs were stimulated with 10, 20 and 40 µg/mL of each extract, and 7.5, 15 and 30 µM of pure compounds. Cell viability, respiratory burst assay (RBA), nitric oxide synthase (NOS) and lysozyme activity were analysed after 24 h of stimulation. The results indicated that hexane fraction of both plants extracts inhibited the viability of HKLs. In addition, dichloromethane fraction of Pg extract and ethyl acetate fractions of both plants enhanced the cell viability compared to control at 24 h. Dichloromethane and ethyl acetate fractions of Pg considerably enhanced the RBA and NOS production in HKLs. Similarly, RBA level was significantly increased in HKLs stimulated with Pg hexane (20 and 10 µg/mL) and aqueous (10 µg/mL) fractions. However, the NOS production was dose-dependently inhibited in HKLs treated with Pa ethyl acetate and both plants aqueous fractions. The lysozyme activity was significantly increased in cells treated with Pa aqueous and non-tannins fractions and Pg crude extract as well as all fractions except the aqueous one. Concerning the pure compounds, Cor, Gua, Urs and Hyp differentially inhibited the HKLs viability. All pure compounds significantly stimulated, on a dose dependent manner, the RBA production as well as the lysozyme activity in HKLs. On the other hand, the NOS production was significantly reduced in HKLs treated with Ole (30 µM) and Hyp (7.5 µM). These results highlighted that the crude ethanol extracts of *P. guajava* and *P. amarus*, their fractions and some of their pure components at certain concentration can potentially act as immunomodulators, and could be considered as valuable candidates in fishery sciences.

1. Introduction

Strengthening immunity is one of the promising strategies to control infectious diseases in aquaculture. In aquatic animals, the innate immunity is mainly developed, probably in compensation to the rather limited adaptive immunity when compared to higher vertebrates (Warr, 1995, Du Pasquier *et al.*, 1998). Mononuclear phagocytes including monocytes and macrophages are the key players in the immune system and other cellular processes such as lymphocyte proliferation, inflammatory and apoptosis (Secombes and Fletcher, 1992, Yang *et al.*, 2013). To destroy and engulf foreign pathogens, macrophages will release antibacterial substances including reactive oxygen species (ROS), which are measured by respiratory burst assay, and reactive nitrogen species via phagocytosis process (Sharp and Secombes, 1993, Vallejos-Vidal *et al.*, 2016). Ulvestad et al., (2018) reported that both immunostimulants including bacterial lipopolysaccharide (LPS) and ß-glucan could stimulate the ROS production in Atlantic salmon (*Salmo salar*) macrophages (Ulvestad *et al.*, 2018). Similarly, the ROS activity was also increased in Longfin yellowtail *Seriola rivoliana* leukocytes treated with phenolic compounds from hawthorn *Crataegus mexicana* nano-encapsulated with maltodextrin (Reyes-Becerril *et al.*, 2019). Monocytes and macrophages also play a vital role in producing lysozyme, although the latter was recorded with a higher level of lysozyme (Lewis *et al.*, 1990). It was indicated that tumor necrosis factor-α (TNF-α) could regulate the production of lysozyme in monocytes and macrophages, while neutrophils are responsible for the release of lysozyme (Bajaj *et al.*, 1992, Manicourt *et al.*, 1993).

Natural products have been applied as a promising source for medicinal discovery, because they may be effective to control various diseases and mitigate many side effects that are associated with synthetic drugs. Among plants of potential medical interest, guava *Psidium guajava* L. and bhumi amla *Phyllantus amarus* Schum and Thonn are known to possess pharmacological activities including anti-bacterial, anti-oxidant as well as immune response activities. *P. guajava* leaf extract significantly improved the growth performance, mucosal and serum immunity as well as antioxidant activities in rohu *Labeo rohita* or Mozambique tilapia *Oreochromis mossambicus* (Giri *et al.*, 2015, Gobi *et al.*, 2016a, Fawole *et al.*, 2016). Indeed, the guava leaf extract could stimulate the production of NOS and ROS in common carp *Cyprinus carpio* var. *koi* L (David *et al.*, 2017), RBA and lysozyme activity in rohu *Labeo rohita* (Hamilton) (Fawole *et al.*, 2016); whereas the mRNA expression of *iNOS* significantly decreased in rohu fed with guava extract (Giri *et al.*, 2015). Moreover, several *in vitro* studies also demonstrated that *P. amarus* and *P. guajava* extracts could attenuate LPS induced proinflammatory response in RAW 264.7 cells (Choi *et al.*, 2008) and U937 macrophages (Harikrishnan *et al.*, 2018), respectively. Additionally, flavonoids isolated from *P. guajava* as well as phyltetralin, niranthin and nirtetralin identified from *P. amarus* were also revealed to possess anti-inflammatory activity via the inhibition of LPS induced cytokines such as *TNFα*, *IL1β*, *IL10*, *iNOS*, and *COX*-2 (Kassuya *et al.*, 2005, Sen *et al.*, 2015). Ilangkovan et al. (2013) also suggested that phyllanthin and hypophyllantin strongly suppressed phagocytic activity in human leucocytes (Ilangkovan *et al.*, 2013). After a large *in vitro* screening of 20 commonly used traditional plant extracts (using striped catfish leukocyte models isolated from blood and head kidney) followed by an *in vivo* validation, we previously found that *P. amarus* and *P. guajava* ethanol extracts potentially modulated immune endpoints and better protected striped catfish against the infection by the bacteria *Edwardsiella ictaluri* (Nhu *et al.*, 2019a, Nhu *et al.*, 2019b).

Our earlier studies focused on the immunomodulatory effects of *P. amarus* and *P. guajava* crude ethanol extracts on the striped catfish. However, to try to identify the type of compound(s) which could explain this activity, we fractionated these crude extracts with different polarity solvents to separate them in fractions containing different levels of bioactive compounds (Truong *et al.*, 2019), which may result in various effects on the immune functions in fish. Nowadays, plant-based natural sources are rapidly developing as commercial products due to the increasing demand for sustainable applications in aquaculture. Hence, new insights on chemical characterization as well as analysis of their biological activities are necessary to improve our knowledge , standardize active extracts and activities, and determine the mode of action/indications. In the present study, we aimed to investigate the immunomodulatory effects of crude ethanol extracts of *P. amarus* and *P. guajava* as well as their fractions including n-hexane, dichloromethane, ethyl acetate, water and non-tannin ones, using striped catfish head kidney leukocyte model. On the other hand, we also tried to evaluate the phytochemical constituents of the *P. amarus* and *P. guajava* crude ethanol extract as well as assess their effects on lysozyme, RBA and NOS activities in striped catfish head kidney leukocytes.

2. Material and methods

2.1. Chemicals

All organic solvents (VWR, Belgium) *n*-hexane, dichloromethane, ethyl acetate, methanol, ethanol used for sample extraction and fractionation were of analytical grade. Methanol, acetonitrile, formic acid were HPLC grade and water had MilliQ quality. The standards of ellagic acid, hyperin, isoquercitrin, guajaverin, avicularin, asiatic acid, corosolic acid, maslinic acid, oleanolic acid, ursolic acid were purchased form Sigma, Belgium whie hypophyllanthin was obtained from Extrasynthese (Genay – France). The mixture of four triterpenic ester (4TTE) including 3*β*-O-(*cis*-*p*-coumaroyl)corosolic acid, 3*β*-O-(*trans*-*p*-coumaroyl)corosolic acid, 3*β*-O-(*cis*-*p*-coumaroyl)maslinic acid, and 3*β*-O-(*trans*-*p*-coumaroyl)maslinic acid were isolated by Lucy Catteau as explained by André et al (2019) and the purity of this mixture was measured as 93% using an Accela HPLC system (Thermo Fisher Scientific).

2.2. Plant material

Leaves of *Psidium guajava* L. were collected at Binh-Minh district, Vinh-Long province, Vietnam and aerial parts of *Phyllanthus amarus* Schun and Thonn were collected at Thot-Not district, Can-Tho city, Vietnam and identified by PhD. DANG Minh Quan (Department of Biology Education, School of Education, Can Tho University). Voucher specimens of Psig2017, Phya2017 respectively were deposited at Department of chemistry, College of Natural Sciences, Can Tho University.

2.3. Preparation of plant extracts and fractions

The dry powders of *Psidium guajava* leaves and *P. amarus* aerial parts (1 kg) were macerated three times with ethanol (5 L) at room temperature, then filtered by filter-paper. The solutions were concentrated under reduced pressure with a rotatory evaporator at 45°C and then lyophilized to give 114 g (Pg) or 133 g (Pa) crude extracts. 530 mg Pg crude extract were suspended in water (60 mL) and fractionated using liquid-liquid partition three times with 20 mL of hexane, dichloromethane, ethyl acetate respectively to obtain 4 fractions: hexane (66 mg), dichloromethane (75 mg), ethyl acetate (94 mg) and aqueous fractions (210 mg). 6g of Pa crude extract was suspended in 700 ml of water and then fractionated using liquid-liquid partition with 3 x 250 mL of hexane and ethyl acetate respectively to give 3 fractions: hexane (1 g), ethyl acetate (600 mg) and aqueous fractions (4 g).

Elimination of tannins of crude extract of *P. amarus*

Twenty mg of crude Pa extract were suspended in water and applied on a 5 g polyamide column in order to eliminate the tannins. The column was eluted with 10 mL water, 10 mL of 50% of MeOH/water and then 40 mL of MeOH. The obtained solutions were combined and evaporated under reduced pressure to furnish 13 mg of fraction Pa-F4.

All samples were stored at 3-5°C prior to be used for the immunomodulatory effects test and for identification of active compounds by HPLC-DAD-orbitrapMS for Pg and HPLC-UV for Pa.

The extracts and fractions were re-dissolved in dimethyl sulfoxide (DMSO, Saint Louis, MO, US) in order to prepare stock solutions at 8, 4 and 2 mg/mL for *in vitro* study. The stock solutions were stored at – 20 °C until used.

2.4. Analysis of samples by HPLC-DAD-Orbitrap-MS

Analyzes were performed on an Accela HPLC system (Thermo Fisher Scientific) consisting of a PDA detector connected with a Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer from the UCLouvain Massmet platform. HR-MS were measured with APCI source in the negative mode using full-scan MS with a mass range of 100-2000 *m/z*. The following (-) APCI conditions were applied: vaporizer temperature, 400°C; sheath gas (N_2) flow rate, 25 a.u.; auxiliary gas (N_2) flow rate, 25 a.u.; sweep gas (N_2) flow rate, 5 a.u.; capillary temperature, 250°C; capillary voltage, 10 V; tube lens, 125 V.

Identification of phenolic compounds was done on a Phenomenex® Lichrospher C18, 4.6 x 250 mm column packed with 5 µm particles. 10 µL of samples at 1 mg/mL concentration were injected in the full loop injection mode. The column was eluted at a constant flow rate of 0.8 mL/min using a binary solvent system: solvent A, MilliQ water 0.1% formic acid and solvent B, acetonitrile HPLC grade (0-10 min, 17% B; 28 min, 28% B; 38 min, 38% B; 39-49 min, 100% B). DAD-UV detector was set at 254 nm.

The column chosen for analysis of triterpenic acids was an Agilent® Poroshell 120 EC-C18, 4.6 x 100 mm packed with 2.7 µm particles. 20 µL of samples (1 mg/mL) were injected in the full loop injection mode. A flow rate of 0.4 mL/min was applied for this method and the mobile phase consisted of a gradient of solvent A, MilliQ water; solvent B, acetonitrile HPLC grade and solvent C, methanol HPLC grade (0-3 min, 35% A, 30% B and 35 % C; 30-48 min, 0% A, 65% B and 35% C). DAD-UV detector was set at 210 nm and the LC-orbitrap conditions were the same as previously mentioned.

2.5. Analysis of samples by HPLC-UV

The crude *P. amarus* extract, as well as its fractions and pure hypophyllanthin were analysed by HPLC-UV using the method described by Jantan et al. (2014) on a Hitachi Merck® equipment with a L6200 intelligent pump and UV L4000 Merck® detector. The column was a RP18 Licrospher® (5 mm, 250 mm×4.5 mm). The flow rate was 0.4 mL/min. The mobile phase consisted of MilliQ water 0.1% orthophosphoric acid (A) and acetonitrile (B) (0 min, 5% B, 20-35 min, 95% B, 36-50 min, 100% B). UV detector was set at 205 nm.

2.6. Experimental fish

For *in vitro* experiments, striped catfish juveniles of 50± 5 g were acclimated to laboratory conditions for 15 days at 28±2 °C in composite tank (2000 L). Fish were fed twice (9 am and 3 pm) daily at a feeding rate of 1% of body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco) under a natural photoperiod prior to their use in the *in vitro* assay. The health status of experimental fish was checked following the method described in the previous study (Nhu *et al.*, 2019b). Healthy fish which did not present abnormal clinical and pathogenic bacteria, were used for experiment.

2.7. Isolation of head kidney leukocytes (HKLs)

Head kidney tissue was aseptically excised from freshly euthanized striped catfish and gently pushed through a 40-µm nylon mesh (VWR International, LLC, Radnor, PA USA) with L-15 medium (pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) supplemented with a 1% solution of 10,000 µg/mL streptomycin +10,000 U/mL penicillin (Invitrogen). After washing with PBS 1X, the residual erythrocytes in HKLs were removed by incubating in 5 min with an osmotic shock sterile red blood cell lysis buffer (pH 7.4). The suspension was neutralized by PBS 1X (v: v) and centrifuged as indicated previously, then the leukocytes were collected and suspended in L-15 medium supplemented with 5% fetal bovine serum (FBS; Invitrogen), 1% Hepes (20 mM, Sigma, USA) and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A (PhA M form, Invitrogen). Viable cells were adjusted to 5×10^6 cells/mL after enumeration using trypan blue stain (VWR, Leuven, Belgium) and seeded in wells of a 24 or 48-well plate (Greiner Bio-One, Vilvoorde, Belgium).

2.8. Stimulation of primary HKLs

After isolation of striped catfish HKLs, 2 mL of cell suspension (5×10^6 cells/mL) in L-15 medium supplemented with 5% FBS, 1% Hepes and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A were added to each well of 24-well plate (Greiner Bio-One, Vilvoorde, Belgium). Afterward, leukocytes stimulation was carried out with at 10, 20 and 40 µg/mL for each crude extract and fraction or at 7.5, 15, and 30 µM for each pure compound (table 1). Cells cultivated in the same medium containing 0.5% DMSO served as control. Each experiment was conducted in triplicates. The HKLs were incubated at 28 °C in a humidified atmosphere of 5% CO_2.

2.9. Viability test with extract fractions and pure compounds

Cell viability was determined after stimulation with at 10, 20 and 40 µg/mL for each crude extract and fraction or at 7.5, 15, and 30 µM for each pure compound during 24 h with different concentrations of extract fractions and pure compounds. Each treatment was triplicate. The determination was done by a MTS test following the manufacturer's protocol. Briefly, 20 µL of MTS test reagent solution (CellTiter 96® Aqueous One Solution Reagent, Sigma, USA) was added to the cells in culture medium (100 µL) (after exposure to different doses of extracts, fractions, or pure compounds for 24 h in a 96-well plate); a measurement of absorbance at 490 nm was then carried out after 4 h of incubation at 28 °C. The cell viability was calculated by the percentage ratio between the absorbance of the treated and that of the cell control without extract, considered as 100%.

2.10. Immune variables

The humoral immune response was then assessed for 24 h post stimulation (hps) with the concentrations mentioned above. The cell suspension at each time point was collected for nitric oxide species and respiratory burst assays. The residual cell suspension was collected by centrifugation at 10000 × g at 4 °C in 5 min, the supernatant was used for lysozyme analysis.

Lysozyme assay

The lysozyme assay protocol was adapted from Ellis (Ellis, 1990) and Milla et al. (Milla *et al.*, 2010) for HKLs and serum of striped catfish. In 96-well microplates, the lysozyme activity assay was initiated by mixing 30 µL of cell suspension with 130 µL of lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich, MO, USA) suspension in phosphate buffer, pH 6.2 (0.3 mg/mL). The difference in absorbance at 450 nm was monitored between 0 and 30 min for plasma (0 and 15 min for HKLs) and used to calculate units of lysozyme activity. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

Respiratory burst assay

Respiratory burst was adapted from Rook et al (Rook *et al.*, 1985). HKLs were washed twice in L-15 medium (1000 g, 5 min, 28 °C). The culture media were then replaced by a corresponding fresh medium containing 2 mg ml^{-1} NBT. Cells were incubated during 1 h at 28 °C in a light protected environment. After 1h, the cells were washed twice in PBS and the reaction was stopped by adding 200 µL of methanol. The cells were rinsed by centrifugation (1000 g, 10 min, 4 °C) and finally air dried for 10 min. Resulting formazan was dissolved in 240 µL of KOH 2M and 280 µL of N-dimethylformamide. The absorbance of the final supernatant was measured at 550 nm. A standard curve was done using serial dilutions of NBT directly dissolved in KOH 2M and N-dimethylformamide. Samples and negative control without cells were performed in duplicates. The activity was reported on protein concentration in cell suspension measured by Bradford assay.

Nitric oxide species assay

Production of NOS was measured by the Griess reaction. First, 100 µL of cell suspension were incubated with 5 µL of *Edwardsiella ictaluri* suspension (OD 2) resuspended in corresponding culture media during 1 h at 28°C. Then, 100 µl of Griess reactant was added and solutions were incubated for 15 min. The absorbance was measured at 540 nm. Standard straight line was performed by using serial dilutions of $NaNO_3$. Negative control corresponded to culture media (without cells) incubated with *E. ictaluri* suspension and Griess reactant. The activity was reported on protein concentration in cell suspension measured by Bradford assay.

2.11. Statistical analysis

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY:IBM USA). Results are presented as mean ± standard deviation (S.D.). One-way analysis of variance was run to find out any difference in immune parameters in treated cells compared to control cells.

3. Results

3.1. Chemical constituent identification of Phyllanthus amarus by HPLC-UV

The HPLC-UV profiles of the crude extract and fractions of *P. amarus* were analysed according to Jantan et al (2014). The chromatograms are given on Fig. 1. They show that crude extracts before and after removing tannins contain hypophyllathin, as well as the hexane fraction.

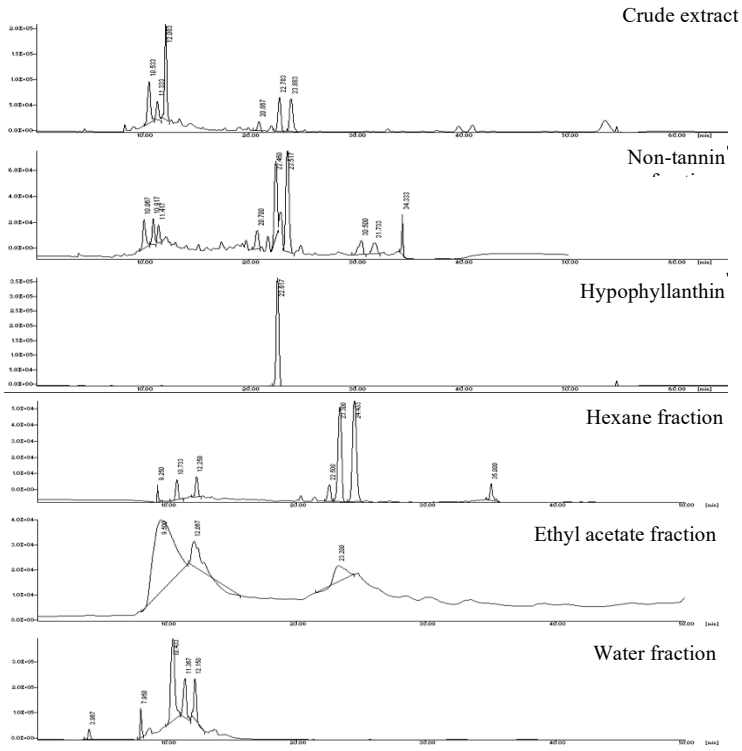


Figure 1. HPLC-UV (205 nm) profiles of the crude *P. amarus* extracts with and without tannins, hypophyllanthin and hexane, ethyl acetate and water fractions from its crude ethanolic extract.

Table 1. List of extracts, fractions and pure compounds were purification from *P. amarus* and *P. guajava*

N^0	NAME	EXPLANATION
1	Pg extract	Crude extract of *Psidium guajava*
2	Pg-F1	Fraction *n*-hexane of *Psidium guajava*
3	Pg-F2	Fraction CH_2Cl_2 of *Psidium guajava*
4	Pg-F3	Fraction ethyl acetate of *Psidium guajava*
5	Pg-F4	Fraction H_2O of *Psidium guajava*
6	Pa extract	Crude extract of *Phyllanthus amarus*
7	Pa-F1	Fraction *n*-hexane of *Phyllanthus amarus*
8	Pa-F2	Fraction ethyl acetate of *Phyllanthus amarus*
9	Pa-F3	Fraction H_2O of *Phyllanthus amarus*
10	Pa-F4	Non tannin fraction of *Phyllanthus amarus*
11	Avicularin- Avi	Found in crude extract of *Psidium guajava*
12	Guajaverin- Gua	Found in crude extract of *Psidium guajava*
13	Corosolic acid- Cor	Found in crude extract of *Psidium guajava*
14	Ursolic acid- Urs	Found in crude extract of *Psidium guajava*
15	Olenanolic acid- Ole	Found in crude extract of *Psidium guajava*
16	Hypophyllanthin	Found in crude extract of *Phyllanthus amarus*

3.2. Chemical constituent identification of Psidium guajava by HPLC-DAD-Orbitrap-MS

To determine the phytochemical composition of the ethanol extract of *P. guajava* leaves collected in Vietnam, HPLC-DAD-Orbitrap-MS chromatographies, using two different mobile phases were used to identify the major components from the crude extract and their fractions. The results showed that two major groups of metabolites were present: phenolic and triterpenic compounds. The chromatograms of these two main bioactive groups are shown in **Fig.** 2 and **Fig.** 3 respectively. These compounds were identified based on characteristic properties such as the retention times, UV absorption spectra, experimental mass data, best matching molecular formula provided by the Xcalibur software of the Orbitrap-MS fragments and comparison with standards. Compound 4, for which no standard was available, is an isomer of guajaverin and avicularin, having the same UV spectra and MS molecular ion of $[M-H]^-$ 433.07779, in accordance with the formula $C_{20}H_{18}O_{11}$. It was identified as reynoutrin because of the presence of characteristic fragments in MS, its UV absorption spectra and comparison with published data (Díaz-de-Cerio *et al.*, 2016, Wang *et al.*, 2017, Bezerra *et al.*, 2018). Unknown compound **7** possessed the same mass fragments and UV spectrum than quercetin or morin, but comparison of retention times with the quercetin and morin standards showed that they were different compounds. Thus, compound 7 was suggested to be an isomer of quercetin.

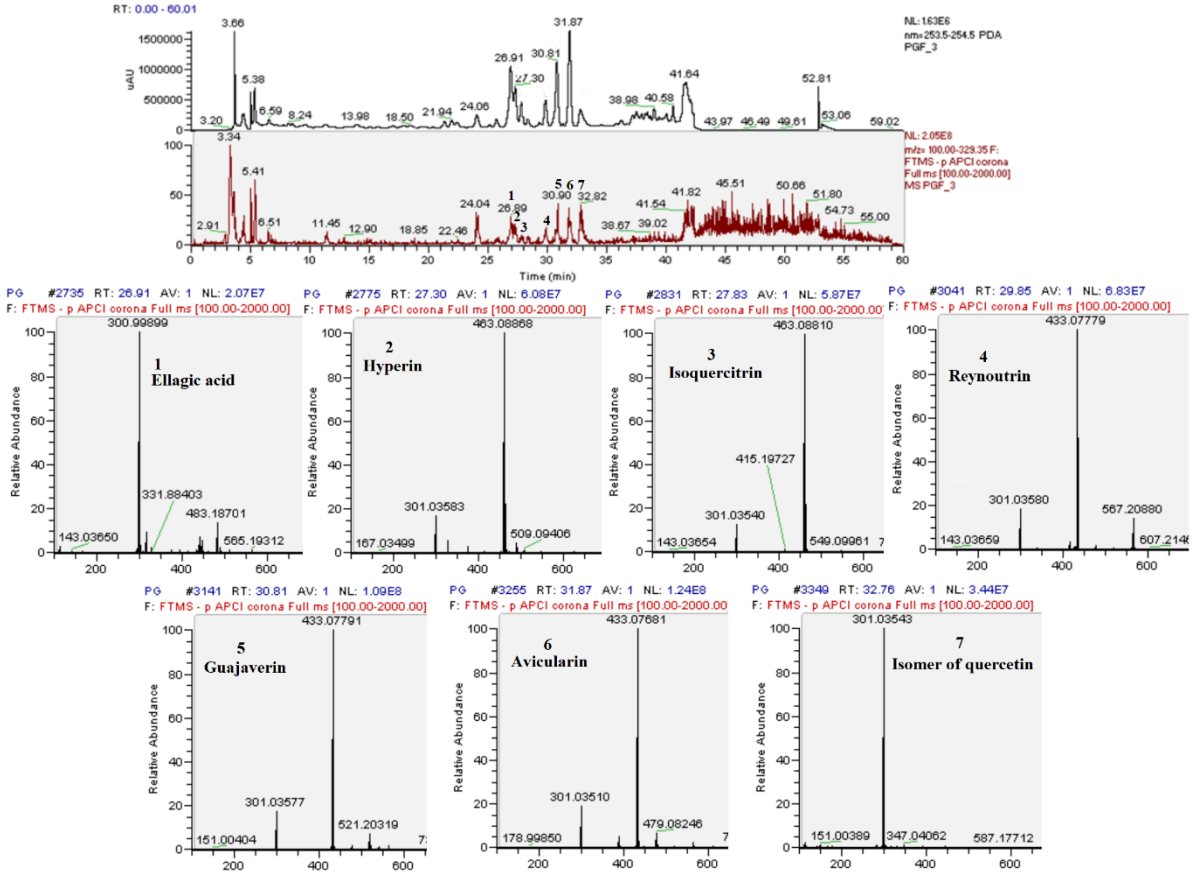


Figure 2. HPLC-DAD (upper) and HPLC-Orbitrap-MS-TIC (lower) chromatograms of the crude ethanolic extract of *P. guajava* with the method used to analyse phenolic compounds and mass spectra of identified phenolic compounds in negative ion mode.

Identified compounds are summarized in Table 1, Table 2 and Table 3.

Table 2. Identification of phenolic compounds from *P. guajava* leaves using HPLC-DAD-Orbitrap -MS method

Peak	Retention time (min)	λ_{max} (nm)	Molecular ion (m/z) $[M-H]^-$	Mw	Formula	Error (ppm)	Compounds
1	26.91	254, 367	300.99899	302	$C_{14}H_6O_8$	1.096	Ellagic acid
2	27.30	255, 354	463.08868	464	$C_{21}H_{20}O_{12}$	1.578	Hyperin
3	27.83	256, 353	463.08810	464	$C_{21}H_{20}O_{12}$	0.998	Isoquercitrin
4	29.85	255, 353	433.07779	434	$C_{20}H_{18}O_{11}$	1.252	Reynoutrin
5	30.81	255, 353	433.07791	434	$C_{20}H_{18}O_{11}$	1.372	Guajaverin
6	31.87	255, 350	433.07681	434	$C_{20}H_{18}O_{11}$	0.272	Avicularin
7	32.76	255,350	301.03543	302	$C_{15}H_{10}O_7$	1.151	Unknown

Table 3. Identification of triterpenic compounds from *P. guajava* leaves using HPLC-DAD-Orbitrap-MS method

Peak	Retention time (min)	λ_{max} (nm)	Molecular ion (m/z) $[M-H]^-$	Mw	Formula	Error (ppm)	Compounds
1	13.81	203	487.34351	488	$C_{30}H_{48}O_5$	1.709	Asiatic acid
2	23.79	204	471.34830	472	$C_{30}H_{48}O_4$	1.414	Maslinic acid
3	24.47	205	471.34808	472	$C_{30}H_{48}O_4$	1.194	Corosolic acid
4	26.17	204, 307	617.38544	618	$C_{39}H_{54}O_6$	1.774	3*β*-O-(*cis/trans-p*-coumaroyl)maslinic acid
5	26.81	204, 307	617.38446	618	$C_{39}H_{54}O_6$	0.794	3*β*-O-(*cis/trans-p*-coumaroyl)corosolic acid
6	27.44	205, 311	617.38519	618	$C_{39}H_{54}O_6$	1.524	3*β*-O-(*trans/cis-p*-coumaroyl)maslinic acid
7	28.08	205, 311	617.38568	618	$C_{39}H_{54}O_6$	2.014	3*β*-O-(*trans/cis-p*-coumaroyl)corosolic acid
8	30.13	205	455.35333	456	$C_{30}H_{48}O_3$	1.358	Oleanolic acid
9	30.43	205	455.35397	456	$C_{30}H_{48}O_3$	1.998	Ursolic acid

The major phenolic compounds from the crude extract of *P. guajava* (see Fig. 2 and Table 2) were ellagic acid (Díaz-de-Cerio *et al.*, 2016, Bezerra *et al.*, 2018), hyperin (Díaz-de-Cerio *et*

al., 2016), isoquercitrin (Díaz-de-Cerio *et al.*, 2016, Bezerra *et al.*, 2018), reynoutrin (Díaz-de-Cerio *et al.*, 2016, Wang *et al.*, 2017, Bezerra *et al.*, 2018), guajaverin (Díaz-de-Cerio *et al.*, 2016, Wang *et al.*, 2017, Bezerra *et al.*, 2018), avicularin (Díaz-de-Cerio *et al.*, 2016, Bezerra *et al.*, 2018) which were already identified from other samples of leaves of *P. guajava*. The structures of these compounds are given in **Fig.** 4. HPLC-DAD-UV of the crude extract, the four fractions and the standards were performed to analyse the presence of these phenolic compounds in the different fractions of crude leaves extract of *P. guajava*. The results (**Fig.** 6 and **Fig**. 3) showed that these phenolic compounds were mainly present in the ethyl acetate fraction.

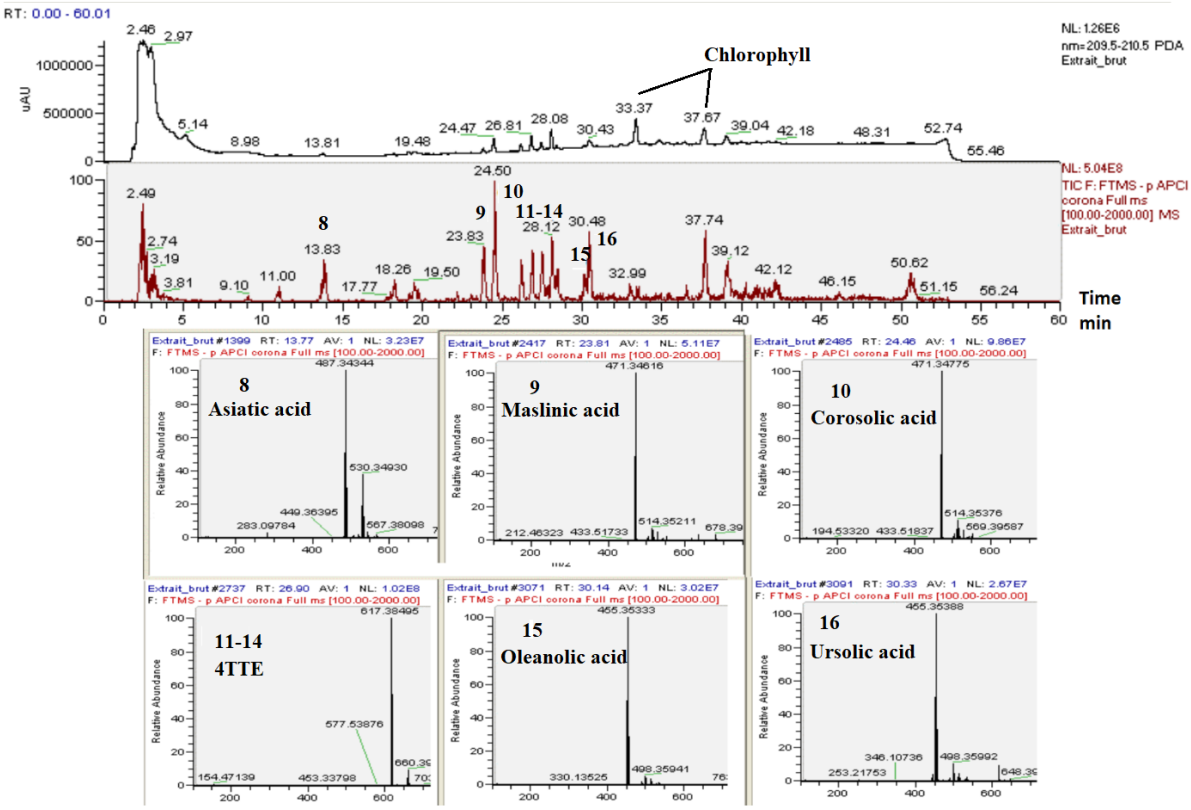
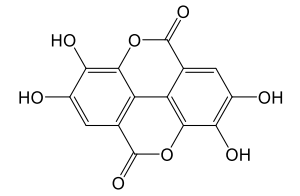
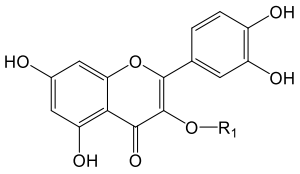


Figure 3. HPLC-DAD (upper) and HPLC-Orbitrap-MS-TIC (lower) chromatograms of the crude ethanolic extract of *P. guajava* with the method used to analyse triterpenic compounds and mass spectra of identified triterpenic compounds in negative ion mode.



1 Ellagic acid



		R_1
2	Hyperin:	galactopyranoside
3	Isoquercitrin:	glucopyranoside
4	Reynoutrin:	xylopyranoside
5	Guajaverin:	arabinopyranoside
6	Avicularin:	arabinofuranoside

Figure 4. Structures of phenolic compounds identified form *P. guajava* leaves.

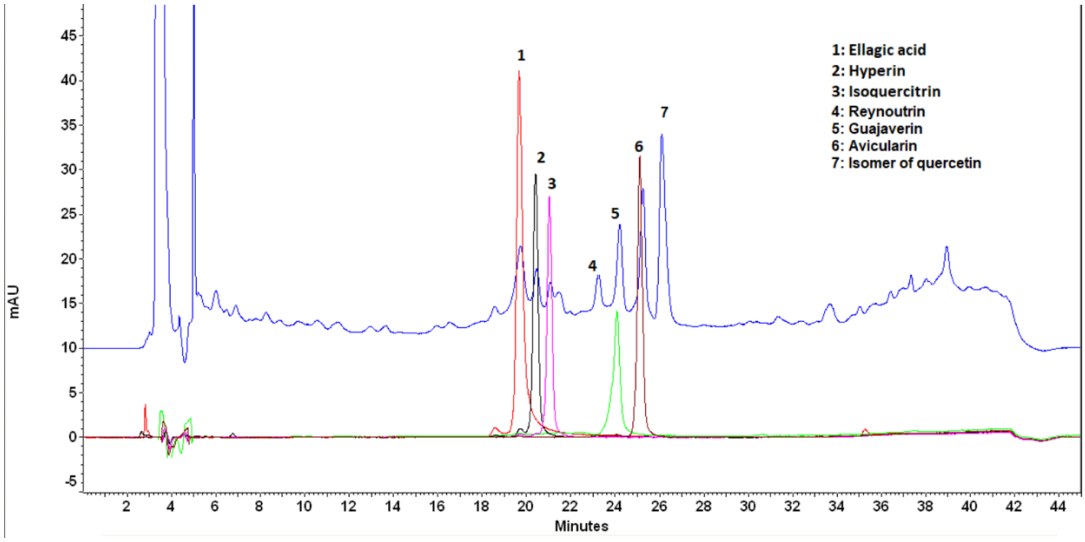


Figure 5. HPLC-DAD-UV chromatograms of crude ethanolic extract of *P. guajava* with the method used to analyse phenolic compounds and the standards of ellagic acid, hyperin, isoquercitrin, guajaverin and avicularin at 254 nm.

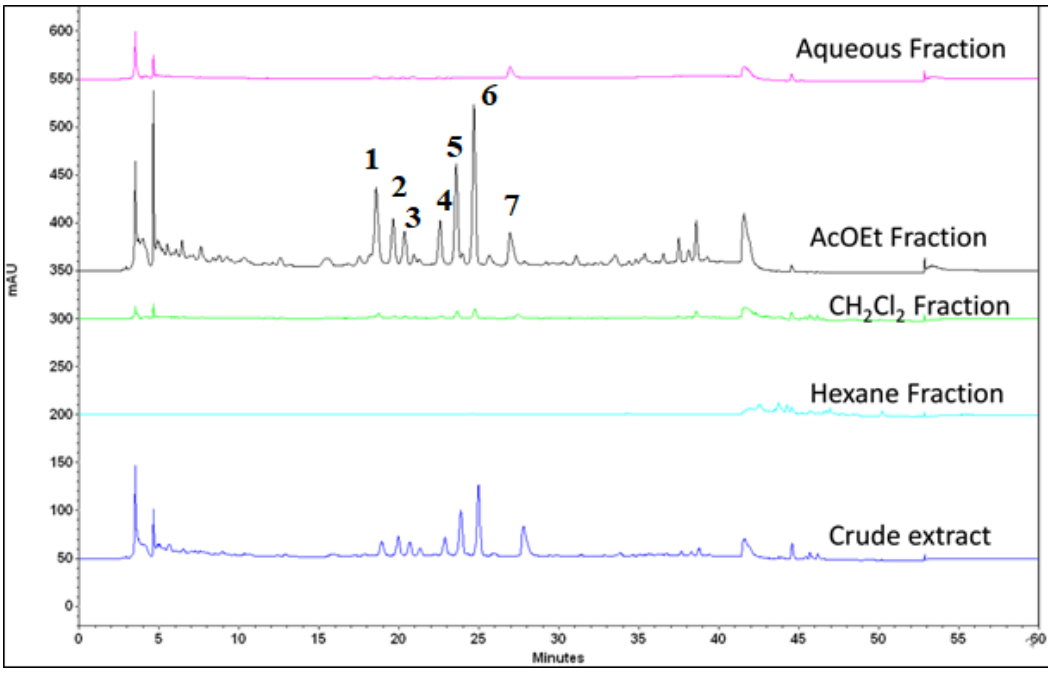
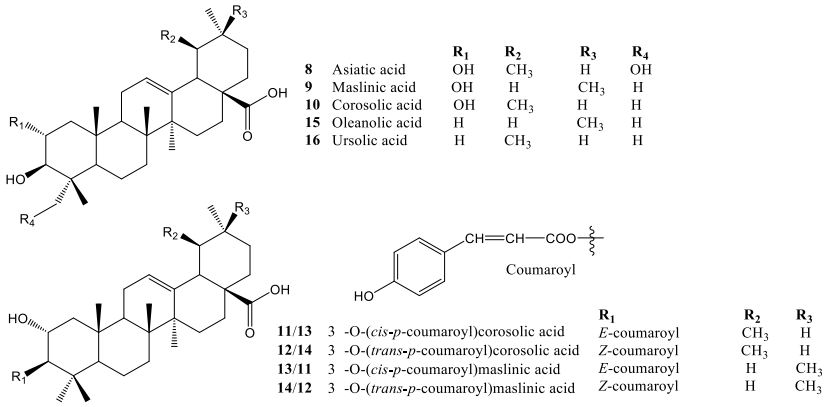


Figure 6. HPLC-DAD-UV chromatograms of the crude ethanolic extract of *P. guajava* and its four fractions with the method used to analyse phenolic compounds at 254 nm.

The results of **Fig.** 5 and Table 3 show that the major triterpenic derivatives from the leaves of *P. guajava* were asiatic acid (Begum *et al.*, 2002), maslinic acid (Metwally *et al.*, 2011), corosolic acid (Begum *et al.*, 2002), 4TTE (3*β*-O-(*cis-p*-coumaroyl)corosolic acid, 3*β*-O-(*trans-p*-coumaroyl)corosolic acid, 3*β*-O-(*cis-p*-coumaroyl)maslinic acid, and 3*β*-O-(*trans-p*-

coumaroyl)maslinic acid) (Shao *et al.*, 2012), oleanolic acid (Begum *et al.*, 2004) and ursolic acid (Begum *et al.*, 2004) which were also previously isolated form other samples of this plant and reported in the literature. Structures are given in **Fig.** 7 while **Fig**. 8 shows the HPLC-DAD-UV chromatograms of the crude extract and reference compounds. We also analysed the presence these compounds in the different fractions by the same method (**Fig.** 8 and **Fig.** 9). The results indicated that they were mainly present in the dichloromethane and ethyl acetate fractions , and that the major components in dichloromethane fraction were the triterpenic derivatives. Although these compounds were known and reported in previous publications, this is the first time that an HPLC-DAD-MS method is described to identify these triterpenic derivatives in the leaves of *P. guajava*. Results also show that the hexane fraction contains mainly chlorophyll derivatives and volatile compounds.



		R_1	R_2	R_3	R_4
8	Asiatic acid	OH	CH_3	H	OH
9	Maslinic acid	OH	H	CH_3	H
10	Corosolic acid	OH	CH_3	H	H
15	Oleanolic acid	H	H	CH_3	H
16	Ursolic acid	H	CH_3	H	H

			R_1	R_2	R_3
11/13	3	-O-(*cis*-*p*-coumaroyl)corosolic acid	*E*-coumaroyl	CH_3	H
12/14	3	-O-(*trans*-*p*-coumaroyl)corosolic acid	*Z*-coumaroyl	CH_3	H
13/11	3	-O-(*cis*-*p*-coumaroyl)maslinic acid	*E*-coumaroyl	H	CH_3
14/12	3	-O-(*trans*-*p*-coumaroyl)maslinic acid	*Z*-coumaroyl	H	CH_3

Figure 7. Structures of triterpenic compounds identified form *P. guajava* leaves

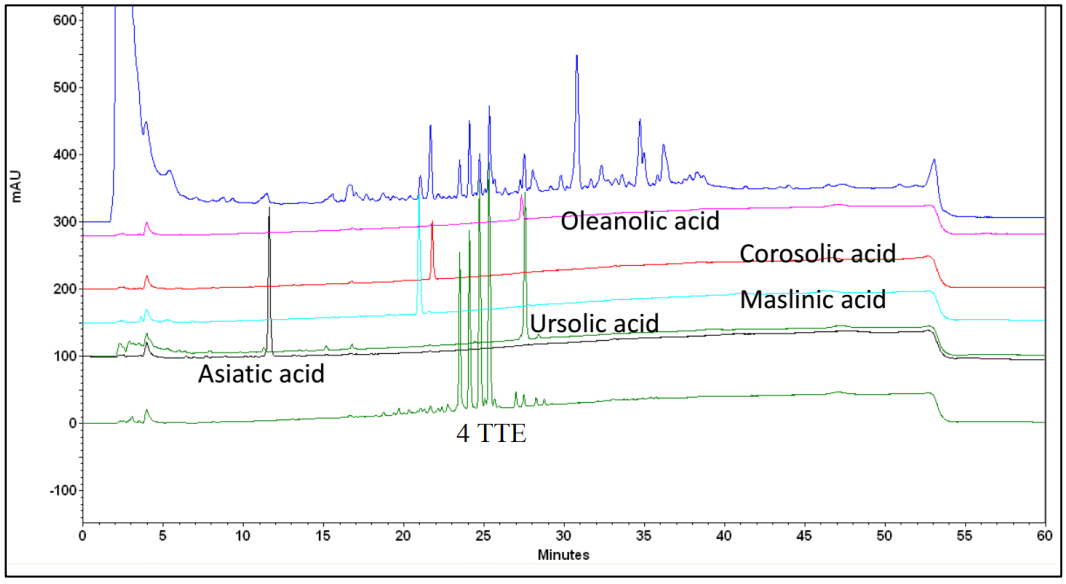


Figure 8. HPLC-DAD-UV chromatograms with the method used to analyse triterpenic compounds of the crude ethanolic extract of *P. guajava* and the standards of asiatic acid, maslinic acid, corosolic acid, 4TTE, oleanolic acid and ursolic acid at 210 nm.

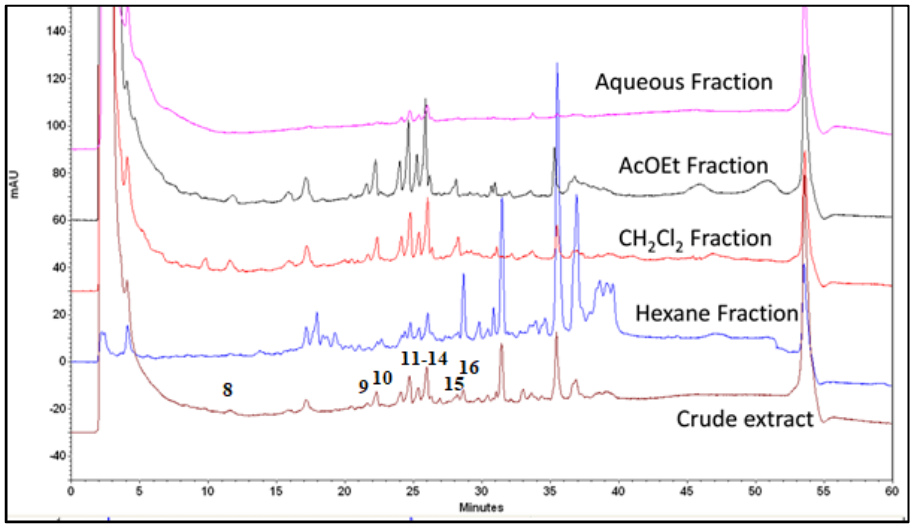


Figure 9. HPLC-DAD-UV chromatograms of the crude ethanolic extract of *P. guajava* and its four fractions with the method of used to analyse triterpenic compounds at 210 nm.

In conclusion, flavonoids and triterpenic derivatives were the major components identified in the crude ethanol extract from the leaves of *P. guajava*, its dichloromethane fraction mainly contained triterpenic derivatives as the main metabolites and its ethyl acetate fraction contained both flavonoid and triperpenic derivatives.

3.3. Effects of extract fractions on immune response of HKLs

3.3.1. Viability

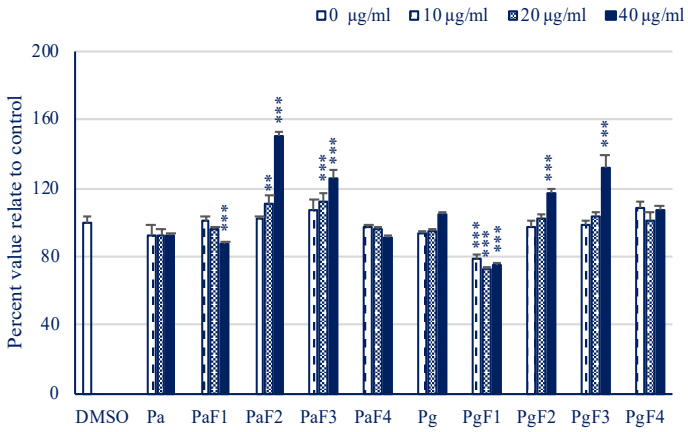


Figure 10. Effect of *P. amarus* and *P. guajava* ethanol extracts and their fractions on viability of striped catfish HKLs after 24h. Pa: *P. amarus*; Pg: *P. guajava*;n-hexane- PaF1 and PgF1; dichloromethane-PgF1; ethyl acetate- PaF2 and PgF3; aqueous- PaF3 and PgF4; and non-tannin fraction- PaF4. Asterisk indicates significant differences in viability levels between stimulated and unstimulated cells at 24h (*$p < 0.05$; **$p < 0.01$; ***<0.001). Values are mean ± S.D. (n = 3).

After stimulation, all tested concentrations of Pa and Pg crude extracts as well as Pa non-tannins fractions and Pg aqueous fractions did not significantly affect the viability of striped catfish HKLs after 24 h (Fig.10). The viability was significantly reduced in cells treated with

Pg *n*-hexane fractions (all concentrations) and Pa *n*-hexane fractions (40 µg/mL) compared to controls. In contrast, high and medium concentrations of Pa ethyl acetate and Pa aqueous fractions showed higher viability than controls HKLs. Similarly, HKLs growth was also stimulated with the highest concentration of Pg dichloromethane fractions and Pg ethyl acetate fractions after 24 h treatment. Nevertheless, the percentage of remaining cells was always higher than 75%, even for the highest concentrations, showing the low toxicity of these extracts.

3.3.2. Immune parameters

As shown in Fig. 11, the RBA, NOS and lysozyme levels in HKLs treated with most Pg fractions were increased compared to those treated by Pa fractions.

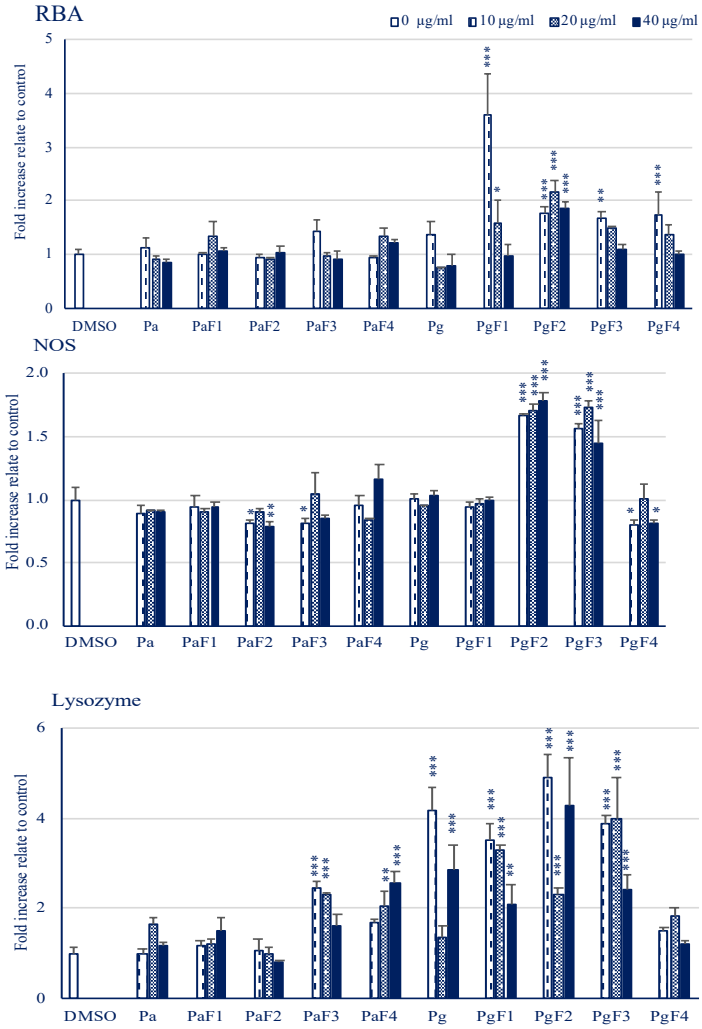


Figure 11. Effect of *P. amarus* and *P. guajava* crude ethanol extracts and their fractions on RBA, NOS and lysozyme activities in striped catfish HKLs at 24h. Pa: *P. amarus*; Pg: *P. guajava*;n-hexane- PaF1 and PgF1; dichloromethane-PgF1; ethyl acetate- PaF2 and PgF3; aqueous- PaF3 and PgF4; and non-tannin fraction- PaF4. Asterisk indicates significant differences in lysozyme levels between stimulated and non-stimulated cells at 24h (*$p < 0.05$; **$p < 0.01$; ***<0.001). Values are mean ± S.D. (n = 3).

3.4. Effects of pure compounds on immune response of HKLs

3.4.1. Viability

After 24 h, all concentrations of Cor and Urs significantly reduced the viability of HKLs compared to control ($p<0.001$) (Fig. 12). Moreover, the highest and medium concentration of Gua, and medium concentration of Hyp also significantly affected the viability of HKLs. However, the viability of HKLs was not affected by Avi and Ole stimulation. Nevertheless, the percentage of remaining cells was always higher than 75%, even for the highest concentrations.

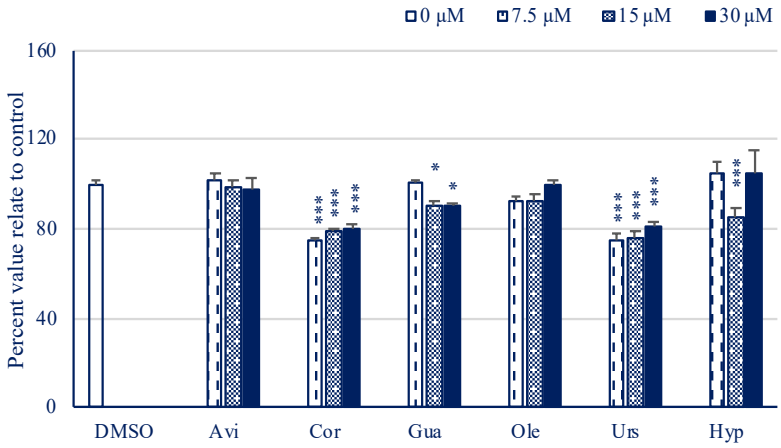


Figure 12. Effect of pure compounds isolated from ethanol extracts of *P. amarus* and *P. guajava* on viability of striped catfish HKLs at 24h. Cor: corosolic acid; Gua: guajaverin; Urs: ursolic acid; Hyp: hypophyllanthin; Avi: avicularin and Ole: oleanolic acid. Asterisk indicates significant differences in lysozyme levels between stimulated and non-stimulated cells at 24h (*$p < 0.05$; **$p < 0.01$; ***<0.001). Values are mean ± S.D. (n = 3).

All concentrations of Pg dichloromethane fraction significantly enhanced the RBA production in HKLs after 24 h contact, while only medium and low concentrations of Pg *n*-hexane fraction, and the lowest concentration of Pg ethyl acetate and Pg aqueous fractions could significantly increase the RBA levels in those cells. Crude Pa and Pg extracts and all Pa fractions did not affect the RBA activity in HKLs after 24 h.

Similarly, all concentration of crude Pa and Pg extracts, Pa *n*-hexane, Pa non-tannins and Pg *n*-hexane fractions did not influence to the NOS production in HKLs, whereas all concentrations of Pg dichloromethane and Pg ethyl acetate fractions significantly increased the NOS level in HKLs compared to control after 24 h. However, the NOS production was significantly suppressed in HKLs treated with high and low concentrations of Pa ethyl acetate and Pg aqueous, as well as with the lowest concentration of Pa aqueous fraction.

Most of the extract fractions differentially modulated the lysozyme activity in HKLs in a concentration dependent manner. Specifically, all concentrations of Pg *n*-hexane, Pg dichloromethane and Pg ethyl acetate fractions significantly stimulated the lysozyme activity in HKLs after 24 h. Several concentrations of Pg extract (highest and lowest) and fractions

including Pa aqueous (medium and lowest), and Pa non-tannins (highest and medium) also significantly enhanced the lysozyme level in HKLs. Moreover, the Pa crude extract, Pa *n*-hexane, Pa ethyl acetate and Pg aqueous fractions did not affect the lysozyme activity in HKLs at 24 h.

3.4.2. Immune parameters

The production of RBA was significantly increased in HKLs treated with all concentrations of Gua ($p<0.001$) (Fig. 13). Moreover, HKLs treated with Cor (7.5 µM), Ole (15 µM) and Urs (30 µM) statistically enhanced their RBA level after 24h ($p<0.05$). On the other hand, RBA was not affected by Avi and Hyp.

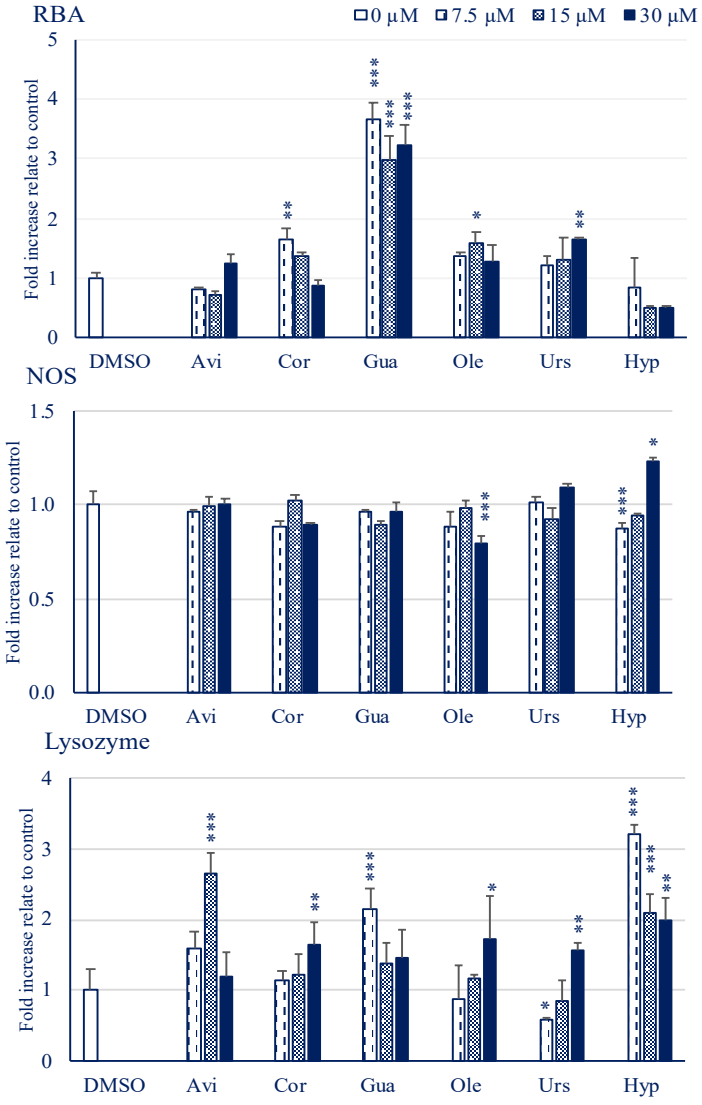


Figure 13. Effect of pure compounds isolated from ethanol extracts of *P. amarus* and *P. guajava* on RBA, NOS and lysozyme activities in striped catfish HKLs at 24h. Cor: corosolic acid; Gua: guajaverin; Urs: ursolic acid; Hyp: hypophyllanthin; Avi: avicularin and Ole: oleanolic acid. Asterisk indicates significant differences in lysozyme levels between stimulated and non-stimulated cells at 24 h (*$p < 0.05$; **$p < 0.01$; ***<0.001). Values are mean ± S.D. (n = 3).

The highest concentration of Hyp significantly enhanced the NOS production in HKLs after 24h stimulation, while the NOS level was statistically decreased in HKLs treated with the lowest dose of Hyp and the highest dose of Ole ($p<0.001$). Several pure compounds including Avi, Cor, Gua and Urs did not affect NOS activity in striped HKLs.

With regards to humoral immune response, all concentrations of Hyp, Avi (15 µM), Gua (7.5 µM), Ole (30 µM), Cor (30 µM) and Urs (30 µM) significantly increased the lysozyme activity in HKLs after 24 h contact, whereas only Urs (7.5 µM) inhibited the lysozyme activity ($p<0.05$).

4. Discussion

Medicinal plants, as well as their components, are well known as alternative natural resources, acting as antimicrobial, anti-stress, growth promotion, appetite stimulation, and immunomodulatory agents (Harikrishnan *et al.*, 2011). In aquaculture, these plant products are usually applied to activate immune response including phagocytic cells, humoral immune response (lysozyme, complement, total immunoglobulin, peroxidase), cellular immune response (RBA, NOS), as well as inflammatory or anti-inflammatory responses, with the ultimate goal to protect fish from infectious pathogens (Mehana *et al.*, 2015) and diseases. Ethanol and methanol solvents are commonly used for plant extraction process. The different polarities of the extracting solvents may cause a wide variation in the level of bioactive compounds (Truong *et al.*, 2019), which may lead to different effective degrees on aquatic animal immune responses. In the present study, we assessed the immunomodulatory effects of crude *P. guajava* and *P. amarus* ethanol extracts and their fractions including n-hexane, dichloromethane, ethyl acetate, and aqueous for Pg and n-hexane, ethyl acetate, aqueous fraction, and non-tannin for Pa on striped catfish head kidney leukocytes. Moreover, major phytochemical constituents of the crude ethanol extracts (Pa and Pg) were identified and examined for their contributions on immune responses of striped catfish head kidney leukocytes.

Crude Pa, Pg extracts and their fractions as well as pure compounds could impact cell morphology, growth, death, and disintegration. In the present study, the viability of striped catfish HKLs was differentially affected according to the dose and type of extract/fraction and compound. Indeed, the *n*-hexane fractions of Pg (40, 20 and 10 µg/mL) and Pa (40 µg/mL) are cytotoxic to HKLs after 24 h of stimulation. In line with these results, Kim et al., (2017) also indicated that the viability of raw 264.7 macrophages decreased after stimulation with *n*-hexane fraction (50 µg/mL) of marsh labrador tea *Ledum palustre* L. at 24 h (Kim, 2017). However, *n*-hexane fraction from *Nerium oleander* L. flower significantly stimulated the proliferation of raw 264.7 macrophages followed by lipopolysaccharide stimulation (100 µg/mL) (Balkan *et al.*, 2018). Our results also highlighted that the dichloromethane and ethyl acetate fractions of Pg, the ethyl acetate and aqueous fractions of Pa could enhance the HKL proliferation after 24 h of stimulation/contact. Cell viability was increased with the increment in concentrations of the fractions. It was already mentioned in the literature that at various concentrations from 0.1 to 10 µg/mL, chloroform, ethyl acetate and aqueous fractions of *Gynura procumbens*

ethanol leaf extract significantly stimulated the proliferative effect in raw 264.7 macrophages after 24 h (Manogaran *et al.*, 2019). In addition, the pure compounds may affect the viability of striped catfish HKLs in a type and/or dose dependent manners. To the best our knowledge, publications about the effects of these pure compounds on leukocytes or macrophages of animals have been limited so far. Several studies have demonstrated the significant reduction in the viability of human retinoblastoma Y-79 and renal carcinoma Caki cells stimulated with corosolic acid (Wang *et al.*, 2018, Woo *et al.*, 2018), in HepG2- human hepatoma and Caco2- human epithelial colorectal adenocarcinoma cell lines stimulated with ursolic acid (Silva *et al.*, 2019). Moreover, the present results observed that the treatment of 15 µM (about 6.5 µg/mL) hypophyllanthin significantly reduced the viability at 24h. In contrast, the viability of human polymorphonuclear leukocytes and monocytes were always higher than 95% after stimulated with ranging concentrations from 0.3125 to 5 µg/mL of hypophyllanthin (Jantan *et al.*, 2014).

Phagocytosis is one of the first steps in the stimulation of the immune response and inflammation (Vatansever *et al.*, 2013). Moreover, ROS are mainly released during phagocytosis (Grayfer *et al.*, 2018), and commonly measured by respiratory burst assay (Baska *et al.*,). In the current study, Pa and its fractions did not affect the RBA production in HKLs. Fractions from Pg including *n*-hexane (20 and 10 µg/mL), dichloromethane (40, 20 and 10 µg/mL), ethyl acetate (10 µg/mL), and aqueous (10 µg/mL) significantly induced the RBA in HKLs after 24 h contact. It could be concluded that Pg extract fractions showed a dose-dependent stimulatory effect on RBA via the activation of NADPH oxidase enzyme in HKLs in HKLs (Klebanoff, 1999). In fish, RBA has been applied as an indicator of innate immune responses (Anderson and Siwicki, 1995, Sahoo and Mukherjee, 2002, Sahoo *et al.*, 2005). Similar increase of ROS production was observed in Mozambique tilapia fed with *P. guajava* ethanol extracts for 30 days (Gobi *et al.*, 2016b). Methanol extract of *P. amarus* hairy root induced the apoptosis in MCF7- cells via the increase of ROS level (Abhyankar *et al.*, 2010). However, both *P. amarus* and *P. guajava* have been shown to possess an anti-inflammatory activity in case of inflammatory activation in mammalian (Díaz-de-Cerio *et al.*, 2017, Seyed, 2019). The present study also indicated that pure compounds isolated from crude ethanol extracts of *P. guajava* including corosolic acid (7.5 µM), guajaverin (30, 15 and 7.5 µM), oleanolic acid (15 µM), and ursolic acid (30 µM) considerably increased the RBA production in HKLs. In agreement with our results, López-García et al. (2015) also demonstrated that triterpenes including oleanolic acid and ursolic acid at 0.625 µg/mL (about 1.4 µM) or 2.5 µg/mL (about 5.5 µM) stimulated the production of ROS in the early phase of mouse macrophage J774 A.1 cell line infected with *Mycobacterium tuberculosis* (López-García *et al.*, 2015). Murine peritoneal macrophages treated with ursolic acid at 4 or 20 µM increased the ROS generation and then released IL-1β protein via the ATP-binding cassette transporter (Ikeda *et al.*, 2007). Moreover, guajaverin is a quercetin derivative (Metwally *et al.*, 2010). In parasites (*Leishmania amazonensis*) infected macrophages, quercetin at 50 µM (Laughton *et al.*, 1989), 100 µM (Lapidot *et al.*, 2002), or 3-12 µM (Fonseca-Silva *et al.*, 2013) could stimulate the increase of ROS production after 72 h and this could explain the increase of ROS production in HKLs treated with guajaverin.

Similar to RBA activity, nitric oxide mediates many physiological and pathophysiological processes including inflammation, and is released via macrophages activation and responsible for killing pathogens (Nathan and Xie, 1994). We presently found that dichloromethane and ethyl acetate fractions from Pg extract significantly enhanced the NOS production in striped catfish HKLs at 24 h. NOS level was also increased in Nile tilapia *Oreochromis niloticus* fed *P. guajava* extract (Omitoyin *et al.*, 2019). However, the NOS production was significantly reduced in HKLs treated with aqueous fraction (40 and 10 µg/mL) from Pg extract, ethyl acetate (40 and 10 µg/mL) and aqueous (10 µg/mL) fractions from Pa extract. Pa extract and its components have been shown to possess inhibitory effects on NOS production in human phagocytes and neutrophils (Ilangkovan *et al.*, 2013, Yuandani *et al.*, 2016). The ethanol/water and hexane extracts of *P. amarus* at different doses could reduce the iNOS and COX-2 levels in mice stimulated with LPS (Kiemer *et al.*, 2003). The various levels of NOS production in HKLs treated with *P. amarus* and *P. guajava* extracts fractions may be due to the presence or absence of flavonoid, triterpenic or phenolic derivatives which were shown to be responsible for stimulating or inhibiting of NOS in the result. On the other hand, oleanolic acid (30 µM) and hypophyllanthin (7.5 µM) significantly inhibited the NOS production, while corosolic acid, guajaverin, avicularin and ursolic acid did not affect NOS production in HKLs. On the contrary, oleanolic acid also enhanced NOS production in J774 A.1 cells infected with *Mycobacterium tuberculosis* (López-García *et al.*, 2015). In RAW 264.7 macrophages, oleanolic acid was reported to possess antitumor activities, and could exhibit a dose dependent increase in *iNOS* and tumor necrosis factor- α transcripts by activating nuclear factor-κB (Choi *et al.*, 2001).

With regard to lysozyme activity, we found that the lysozyme levels in HKLs stimulated by the Pg ethanol extract and its fractions (except aqueous fraction) were stronger than those stimulated by Pa ethanol extract and its fractions. In consistence with these results, we previously also found that the lysozyme activity in HKLs and peripheral blood mononuclear cells (PBMCs) treated with *P. guajava* ethanol extract was considerably enhanced in a dose dependent manner, while *P. amarus* ethanol extract did not affect the lysozyme activity in striped catfish HKLs after 24 h (Nhu *et al.*, 2019b). Lysozyme plays a vital role in fish innate immune response and is mainly produced by macrophages (Lewis *et al.*, 1990, Saurabh and Sahoo, 2008). Our results also indicated that several compounds including avicularin, guajaverin, oleanolic acid and ursolic acid significantly increased the lysozyme activity in HKLs on a dose dependent manner.

Pa extracts with and without tannins as well as the hexane extract were shown here to contain hypophyllathin which is identified as a major active compounds from this plant. We show here that this compound may perhaps be responsible for a part of the effect of Pa non-tannins fraction on lysozyme production by HKLs but that its concentration does not seem to be high enough in other extracts to show activity. Other compounds which were not identified in this work should be responsible for the effects of the aqueous and ethyl acetate fractions.

HKLs contain lymphocytes, monocytes/macrophages and granulocytes (neutrophils, eosinophils, mast cells and basophils) (Samaï *et al.*, 2018). Moreover, ROS, NOS and

lysozyme activities are mainly produced in monocytes/macrophages or granulocytes (Neumann *et al.*, 2001, Esteban *et al.*, 2015., Ogundele, 1998). In the present study, the increment of ROS, NOS and lysozyme activities in HKLs stimulated with the high dose of *P. guajava* ethyl acetate and dichloromethane fractions were accompanied with the viability at 24h, respectively. In contrast, these immune parameters did not consistently increase when compared with the viability in cells treated with the *P. amarus* ethyl acetate and aqueous fractions. Moreover, *P. amarus* ethyl acetate and aqueous fractions siginificantly inhibited the NOS production in HKLs. This may be due to the ethyl acetate and aqueous fractions of *P. amarus* could enhance the lymphocytes proliferation, which mainly involved in the acquired or antigen-specific immune response (Cano, 2013). In agreement with our results, Nworu *et al.*, (2010) *P. niruri* aqueous extract (12.5–200 μg/ml) significantly enhanced the proliferation of T- and B-lymphocytes via the activation of MAPKs, whereas the *P. niruri* extract inhibited NOS production by bone marrow macrophages from treated mice. The relationship between NOS production and T cell proliferation was also demonstrated by Zuo-hua *et al.* (1994), when the decrease of NOS production was followed by the increase of lymphocyte proliferation in spleen cells after being stimulated with garlic derived diallyl trisulfide.

According to the research of David, 2017 (David *et al.*, 2017), flavonoids were identified as possible compounds responsible for the effects of the aqueous leaves extract of *P. guajava* that improved the immune system of the fish *Cyprinus carpio* var. *koi* L, however there was no experiment carried out to test the flavonoid fraction nor pure compounds. Our researches demonstrated that not only the presence of flavonoids, but also triterpenic acids may explain at least partially the immunomodulatory effects of the dichloromethane and ethyl acetate fractions from the leaves extract of *P. guajava*.

Conclusions

The results indicate that *P. guajava* and its fractions, especially dichloromethane and ethyl acetate fractions, shown to contain triterpenic derivatives (both of them) and flavonoids (ethyl acetate fraction) differentially enhanced the RBA and NOS production as well as the lysozyme activity in striped catfish HKLs. On the other hand, several fractions of *P. amarus* including ethyl acetate and aqueous fractions inhibited the NOS production, responsible for the anti-inflammatory effect in HKLs. Compounds including corosolic acid, guajaverin, avicularin, oleanolic acid, and ursolic acid positively enhanced the RBA production and the lysozyme activity in dose and compound dependent manners. It could be concluded that the crude ethanol extracts of *P. guajava* and *P. amarus*, their fractions and pure compounds at certain concentration can potentially act as immunomodulators in striped catfish and could be considered as positive candidates in fishery sciences.

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Chapter 8

Psidium guajava - dichloromethane and ethyl acetate extract fractions early ameliorated striped catfish (*Pangasianodon hypophthalmus*) status via immune response, inflammation and apoptosis pathways

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Hypothesis outlines

The results from the Chapter 7 indicated that the better immune responses were observed in HKLs stimulated with *P. guajava* fractions compared to *P. amarus* fractions. Among the extracts and fractions, dichloromethane and ethyl acetate fractions from *P. guajava* were the best in stimulating the lysozyme, RBA, NOS productions in HKLs. The significant increase of RBA and NOS levels may be the result of activating the inflammatory process. As part of the immune response, inflammation plays an important role in defending the body against pathogens such as viruses, bacteria, and fungi. Our previous chapters did not clearly investigate the mode of action of plant extracts in regulation the fish immune responses, especially in activating the inflammatory responses under the molecular mechanism. In the present study, we are trying to explain how the striped catfish respond to *P. guajava* dichloromethane and ethyl acetate fractions via *in vitro* and *in vivo* tests. The inducible enzymes, including respiratory burst activity- RBA, nitric oxide synthase- NOS, lysozyme, and the different cytokines related to immune responses, inflammatory, and apoptosis process were selected to determine the pathways of these fractions in activating the striped catfish immune responses.

Abstract

Psidium guajava L. has been known to possess immune-modulatory properties on human and other mammals. Although the positive effects of *P. guajava*-based diets on the improvement of immunological status have been established in some fish species, its protective effects underlying molecular mechanisms have not been demonstratedso far. The aim of the study was to evaluate the immune-modulatory effects of two guava extract fractions from dichloromethane (CC) and ethyl acetate (EA) on striped catfish under *in vitro* and *in vivo* experiments. Striped catfish head kidney leukocytes were stimulated with 0, 10, 20 and 40 µg/ml of each extract fraction, and the immune parameters (ROS, NOS and lysozyme) were examined at 6 and 24h post stimulation. The concentrations of each extract fraction at 0, 10 and 40 µg per fish were then intraperitoneally injected to fish. After 6, 24 and 72h of

administration, the immune parameters as well as the expression of some cytokines related to innate and adaptive immune response, inflammation and apoptosis were measured in head kidney. Results indicated that the humoral immune (lysozyme) and cellular immune response endpoints (ROS and NOS) were differentially regulated by CC and EA fractions in dose and time dependent manners in both *in vitro* and *in vivo* experiments. With regards to *in vivo* experiment, CC fraction of guava extract could significantly enhance TLRs-MyD88-NF-κB signaling pathway via upregulation of their cytokine genes (*tlr1*, *tlr4*, *myd88*, *traf6*, and *nfκb2*), followed by the upregulation of inflammatory (*nfκb, tnf*, *il1β* and *il6*) and apoptosis (*tp53* and *casp8*) genes at the early time. Moreover, fish treated with both CC and EA fractions significantly enhanced cytokine genes including *lys* and *inos* at the later time points- 24h or 72h. Our observation suggest that *P. guajava* extracts, especially CC extract fraction, has the potential to modulate the immune response, as well as the inflammatory and apoptotic pathways.

1. Introduction

Guava (*Psidium guajava* L.), is also known as a traditional herbal medicine, has been used not only for food but also for folk medicine (Daswani *et al.*, 2017, Díaz-de-Cerio *et al.*, 2017). A long time ago, many parts of guava including leaves, shoots, bark, flower buds, roots were widely applied to manage several diseases (i.e. diabetes, hypertension, caries, wounds, pain relief and fever) (Gutiérrez *et al.*, 2008). Guava contains various antioxidants and phytochemicals, including essential oils, polysaccharides, minerals, vitamins, enzymes, and triterpenoid acid, alkaloids, steroids, glycosides, tannins, flavonoids and saponins (Smith and Siwatibau, 1975). These bioactive compounds are potentially responsible for bioactivities, including immune stimulation, anti-microbial, anti-inflammatory, anti-cancer as well as anti-oxidant activities (Naseer *et al.*, 2018). In addition, guava is also a plenty source of pectin, an important dietary fiber (García-Betanzos *et al.*, 2017). In the past few years, considerable research has been undertaken to identify and analyze the ingredients of guava (Chaturvedi *et al.*, 2019, Wang *et al.*, 2017, Issa *et al.*, 2016). The guava leaves is considered to be the most active part of guava tree in exploring the medicinal value in pharmaceutical science (Arain *et al.*, 2019, Esfandiari Ghalati *et al.*, 2019, Sharma and Kumawat, 2019, Tella *et al.*, 2019). In recent years, guava was developed as food additive reagents in fish industry, which aimed to improve growth performances, immune responses, antioxidant activity as well as resistance to infectious pathogens (Giri *et al.*, 2015, Gobi *et al.*, 2016, Fawole *et al.*, 2016, David *et al.*, 2017, Nafiqoh *et al.*, 2019, Hoseinifar *et al.*, 2019a, Omitoyin *et al.*, 2019).

One of the potential mechanisms linking animal species and guava intakes may involve attenuation of inflammatory (Díaz-de-Cerio *et al.*, 2017). After inflammation stimulated by lipopolysaccharide (LPS), an ethanol extract from fermented guava leaves (125 μg/mL) significantly inhibited the expression level of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 protein levels via the down-regulation of nuclear factor-κB transcriptional activity (NF- κB) in mouse macrophage cells (Choi *et al.*, 2008). Flavonoid fraction (200 μg/mL) of guava leaf extract could reduce the NF-κB activation in rohu *Labeo rohita* head-kidney macrophages via LPS stimulated (Sen *et al.*, 2015). Moreover, guava also functioned

by stimulating immune system of the cultivated animal. *P. guajava* bark extract induced apoptosis in CCRF-CEM cells via the activation of caspases 3/7, 8 and 9. The production of reactive oxygen species- ROS was also significantly increased in those cells after stimulated with 2 × IC50 (6.35 ± 1.74 µg/mL) guava bark extract 24h (Mbaveng *et al.*, 2018). Guava leaf powder-based diets improved the non-specific immune response in skin mucus and serum as well as significantly upregulated the expression level of interleukin 8 (*il8*) and interleukin 1 beta (*il1β*) in common carp (*Cyprinus carpio*) fingerlings (Hoseinifar *et al.*, 2019b). A previous study of Giri et al., (2015) demonstrated that 0.5% guava leaves extract-based diets could upregulate the levels of *il1β* and tumor necrosis factor alpha (*tnfα*), and downregulate *inos*, *nfkb*, cyclooxygenase-2 (*cox2*), and transforming growth factor beta (*tgfβ*) expression levels in the head kidney, intestine and hepatopancreas of rohu (Giri *et al.*, 2015). These studies revealed a partial molecular basis for the promising immuno-properties of guava extracts.

Among the five ethanol extracts tested, we previously found that *P. guajava* extract-based diets were potentially interesting for modulating immune response and providing better resistance to the pathogenic bacteria *Edwardsiella ictaluri* in striped catfish (*Pangasianodon hypophthalmus*) (Nhu *et al.*, 2019a). To the best of our knowledge, the mechanism of guava leaf extract in fish immune response has not been documented in the literature. Moreover, its biological activities in inflammation are largely unknown in fish. The present study aimed to examine the early functional contributions of different extract fractions including dichloromethane and ethyl acetate from *P. guajava* on striped catfish immune response using *in vitro* and *in vivo* dynamic models. In this objective, we observed the host cell responses to CC and EA extract fractions from *P. guajava* using striped catfish head kidney leukocytes at 6 and 24h- *in vitro* experiment, following an intraperitoneally injection the extracts into striped catfish- *in vivo* experiment. The inducible enzymes, including respiratory burst activity- RBA, nitric oxide synthase- NOS, lysozyme; as well as cytokine genes related to immune response (toll like receptor- *tlr1*, *tlr2* and *tlr4*, myeloid differentiation primary response 88- *MYD88*, tumor necrosis factor receptor associated factor 6- *traf6*, c type lysozyme- *lys*, *inos*, class II major histocompatibility complex-*mhc class II*); inflammatory (*nfκb1* and *nfκb2*, tumor necrosis factor- *tnf*, *il1β* and interleukin 6- *il6*); and apoptosis (*casp3*, *casp8* and tumor protein 53- *tp53*) were selected for *in vivo* experiment.

2. Material and methods

2.1. Extracts preparation

The dry powders of *Psidium guajava* leaves (1 kg) were macerated three times with ethanol (5 L) at room temperature, then filtered by filter-paper. The solutions were concentrated under reduced pressure with a rotatory evaporator at 45°C and then lyophilized to give 114 g (Pg). 530 mg Pg crude extract were suspended in water (60 mL) and fractionated using liquid-liquid partition three times with 20 mL of dichloromethane and ethyl acetate respectively to obtain fractions (dichloromethane and ethyl acetate). The extract fractions were re-dissolved in dimethyl sulfoxide (DMSO, Saint Louis, MO, US) in order to prepare stock solution at 8, 4 and 2 mg/mL for *in vitro* study. The stock solutions were stored in – 20 °C until used.

2.2. Experimental fish

Striped catfish juveniles at 50± 5 g (*in vitro*) and 15±5 g (*in vivo*) were acclimated to laboratory conditions for 15 days at 28±2 °C in composite tank (2000 L). Fish were fed twice (9 am and 3 pm) daily at a feeding rate of 1% of body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco) under a natural photoperiod prior to their use in the *in vitro* assay. The health status of experimental fish was checked following the method described in the previous study (Nhu *et al.*, 2019b). Healthy fish which did not present abnormal clinical and pathogenic bacteria, were used for the experiment.

2.3. *In vitro* experiment

2.3.1. Isolation of head kidney leukocytes (HKLs)

Head kidney tissue was aseptically excised from freshly euthanized striped catfish and gently pushed through a 40-µm nylon mesh (VWR International, LLC, Radnor, PA USA) with L-15 medium (pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) supplemented with a 1% solution of 10,000 µg/mL streptomycin +10,000 U/mL penicillin (Invitrogen). After washing with PBS 1X, the residual erythrocytes in HKLs were removed by incubating during 5 min with an osmotic shock sterile red blood cell lysis buffer (pH 7.4). The suspension was neutralized by PBS 1X (v: v) and centrifuged as indicated previously, then the leukocytes were collected and suspended in L-15 medium supplemented with 5% fetal bovine serum (FBS; Invitrogen), 1% Hepes (20 mM, Sigma, USA) and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A (PhA M form, Invitrogen).Viable cells were adjusted to 5×10^6 cells/mL after enumeration using trypan blue stain (VWR, Leuven, Belgium) and seeded in wells of a 24 or 48-well plate (Greiner Bio-One, Vilvoorde, Belgium).

2.3.2. Stimulation of primary HKLs

After isolation of striped catfish HKLs, 2 mL of cell suspension (5×10^6 cells/mL) in L-15 medium supplemented with 5% FBS, 1% Hepes and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A, were added to each well of 24-well plate (Greiner Bio-One, Vilvoorde, Belgium). Afterwards, leukocyte stimulation was carried out with three doses (10, 20 and 40 µg/mL) of each extract fraction. Cells cultivated in the same medium containing 0.5% DMSO served as control. Each experiment was performed in triplicates. The HKLs were incubated at 28 °C in a humidified atmosphere of 5% CO_2. The humoral immune response as well as cytokine expression were assessed 6 and 24 h post stimulation (hps). At each time point 200 µL of cells were collected for nitric oxide species (NOS) and respiratory burst assays (RBA). The residual cell suspension was collected by centrifugation at 10000 × g at 4 °C in 5 min for gene expression analysis, while the supernatant was used for lysozyme activity testing.

2.4. *In vivo* treatment

After two weeks of acclimation, a total of 150 striped catfish were randomly divided into 4 treated groups and one control group. Fish were intraperitoneally injected with 100 µl of each extract fraction to get to final concentration at 0, 10 and 40 µg/fish. The control treatment was injected with DMSO, each treatment was given in triplicate. The samples were collected at 6,

24 and 72 h for analysis. Briefly, 3 fish per tank (9 fish per treatment) were randomly collected and anaesthetized using 0.1 ppm M222 (Sigma–Aldrich, MO, USA). Blood plasma was individually sampled for lysozyme, fresh spleen was mashed in L-15 medium through a 100 µM nylon mesh and then used for NOS and RBA analysis; while the head kidneys were collected for gene expression analyses.

2.5. Immune variables

2.5.1. The lysozyme assay

The lysozyme assay protocol was adapted from Ellis (Ellis, 1990) and Milla et al. (Milla *et al.*, 2010), which then adapted for HKLs and serum of striped catfish. In 96-well microplates, the lysozyme activity assay was initiated by mixing 10 µL of plasma or 30 µL of cell suspension with 130 µL of lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich, MO, USA) suspension in phosphate buffer, pH 6.2 (0.6 mg/mL for plasma and 0.3 mg/mL for cells). The difference in absorbance at 450 nm was monitored between 0 and 30 min for plasma (0 and 15 min for HKLs) and used to calculate units of lysozyme activity. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

2.5.2. Respiratory burst assay

Respiratory burst was adapted from Rook et al (Rook *et al.*, 1985). Spleen leukocytes as well as HKLs were washed twice in L-15 medium (1000 g, 5 min, 28 °C). The culture media were then replaced by a corresponding fresh medium containing 2 mg ml^{-1} NBT. Cells were incubated during 1 h at 28 °C in a light protected environment. After 1h, the cells were washed twice in PBS and the reaction was stopped by adding 200 µL of methanol. The cells were rinsed by centrifugation (1000 g, 10 min, 4 °C) and finally air dried during 10 min. Resulting formazan was dissolved in 240 µL of KOH 2M and 280 µL of N-dimethylformamide. The absorbance of the final supernatant was measured at 550 nm. A standard curve was done using serial dilutions of NBT directly dissolved in KOH 2M and N-dimethylformamide. Samples and negative control without cells were performed in duplicates. Activity was reported on protein concentration in cell suspension measured by Bradford assay.

2.5.3. Nitric oxide species assay

Production of NOS was measured by the Griess reaction. First, 100 µL of cell suspension were incubated with 5 µL of *E. ictaluri* suspension (OD 2) resuspended in corresponding culture media during 1 h at 28°C. Then, 100 µl of Griess reactant was added and solutions were incubated for 15 min. The absorbance was measured at 540 nm. Standard straight was performed by using serial dilutions of $NaNO_3$. Negative control corresponded to culture media (without cells) incubated with *E. ictaluri* suspension without cells and Griess reactant. Activity was reported on protein concentration in cell suspension measured by Bradford assay.

2.5.4. Immunoregulatory gene expression

For expression analysis, total RNAs were extracted from HKLs and fresh head kidney using Extract-All (Eurobio, Courtaboeuf, France) according to the manufacturer's protocols. Samples were then DNAse treated (DNA-free kit, Ambion, Austin, USA). The extracted RNA was

quantified by spectrophotometry using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The RNA quality was assessed by the 260/280 and 260/230 ratios, while its integrity was evaluated by 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed using RevertAid HMinus First Strand cDNA synthesis Kit (Fermentas, Life Sciences, Germany). The resulting cDNA was diluted 5x for initially testing the efficiency of primers combination or 25x for real-time quantitative PCR.

Table 1. The primers used for realtime PCR analysis

Name	Sequence	Product size	GeneBank Number
Inducible nitric oxide synthase	GGTCTTGAACCAGAGGTCC ACCCAGATGGCTAACCAGGA	103	XM_026935143.1
C typpe lysozyme	ACGCTATGAACGGTGTGAGC CCGGTGTTGTAGTCAGACTCG	120	KU601195
Myeloid differentiation primary response 88	AGGATCGAGGCATCACCGTA GGTTGTAGTCGGTCTGCTCC	129	XM_026938292.1
Toll like receptor 1	CTGGACCATTGCAGTCCCAT CCGTGTCATCTGGCAAGTCT	161	XM_026922777.1
Toll like receptor 2	CCGGAGTTAGAAAGCGCTGA CGAAGTTCTCCGACAGGACG	142	XM_026929176.1
Toll like receptor 4	GCAGGTCCTGGATCTCACAAG CAGGCCAATGTCCACGAGAA	175	XM_026932800.1
Tumor necrosis factor receptor associated factor 6	GGGAGTCGTACCTAAGCCCT TCAGGCAGATGGGACACTCA	135	XM_026937676.1
Class II of major histocompatibility complex	CGCATGCTCAGACTCGGATAA TGAGTCTTGGCGGTCTCGTA	153	XM_026945809.1
Nuclear factor kappa light chain enhancer of activated B cells- 1	ACGTAGAGGTTCAGGAGCGA CTGCTGCGATGTGAAGAGGT	156	XM_026947061.1
Nuclear factor kappa light chain enhancer of activated B cells- 2	TTCCGCAACCCTATGACCAC AGAAGTTCGGCCCATCCAAG	159	XM_026939857.1
Interleukin 1 beta	TTGGCCATGAGTGGCAGATG TCCTGGTCAGTGAACTCCGT	155	XM_026943671.1
Tumor necrosis factor	AGACCAGTCTTTCGCTTCGG CCCTCGGACTCATTATCGGC	129	XM_026942329.1
Interleukin 6	TGATAAGGTTCACCCAACCTCCT TCATGAAGTCTGCGAAGTTGTGC	104	XM_026930094.1
Caspase 8	GGTACCGTGCTAGGGACTGA TCCATCGTGCCTCAACACAC	156	XM_026933210.1
Caspase 3	CTGGCATCGAGGTTGACAGT ACAGGGACTGCATGAACCAC	141	XM_026947691.1
Tumor protein 53	TCCAGGTGCGTGGGAAAGAG GCGATACTTCTCCTGGTCAGC	101	XM_026911853.1
16sRNA	TTACAACTGCCGACCAACGG CCTTAATAGCGGCTGCACCA	134	MF346571.1
Beta-actin	GAAATTGCCGCACTGGTTGTT TGTCTTGGGCGACCCACAAT	110	XM_026929614.1

A total of 2.5 μL of reverse transcription products (diluted 1/25) were used for each real-time PCR. Duplicates were run for each sample. Forward and reverse primers were used at a

concentration of 500 nmol/L and added to Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The relative expression of several immune-related genes was investigated by RT-qPCR, including genes involved in the immune response (*lys*, *inos*, *mhc class II*, *tlr1*, *tlr2*, *tlr4*, *myd88* and *traf6*); inflammatory response *nfκb1* and *2*, *il1β*, *tnf*, *il6*; and apoptosis process (*casp3*, *casp8* and *tp53*). The 16S ribosomal RNA (*16srna*) and beta actin were used as internal control genes (Table 1). These primers were designed on Primer3 software with the primer quality checked using Amplifix software against sequences of common carp published on Genbank. Primer sequences and gene functions are presented in Table 1. The efficiency of each gene was confirmed before analysis. Thermal cycles and fluorescence detection were carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 min of initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. The transcript abundance for each gene was calculated from the threshold cycle (Mbaveng *et al.*) values using their respective standard curve followed by normalization with the geometric mean of ubiquitin and elongation factor. The expression was calculated according to the relative standard curve method of Plaffl (Pfaffl, 2001), where ΔΔCT is $\Delta CT_{treatment} - \Delta CT_{control}$, ΔCT is $CT_{target\ gene} - CT_{housekeeping\ gene}$, and CT is the cycle at which the threshold is crossed. Data are presented as relative fold-change with internal control genes.

2.6. Statistical analysis

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY:IBM USA). The normality of the data and the homogeneity of variance between groups were tested using Shapiro-Wilks and Levene tests. Results are presented as mean ± standard deviation (S.D.) for *in vitro* tested or means ± SEM (standard error of the means) for *in vivo* experiment. One-way ANOVA analysis of variance Duncan's multiple range test at a confidence level of 95% ($p < 0.05$) was used to determine significant differences between immunological variables in fish from the different plant extract treatments and control treatment.

3. Results

3.1. CC and EA extract fractions differentially modulate the RBA, NOS and lysozyme activities in HKLs

In HKLs, both CC and EA extract fractions could differentially enhance the level of RBA, NOS as well as lysozyme activities in a dose and time dependent manners (Fig. 1). All concentrations of EA extract significantly increased the RBA activity compared to control after an early time-6h of stimulation ($p<0.05$), while CC extract did not affect to the RBA activity at this time point ($p>0.05$) (Fig. 1A). Moreover, the highest level of RBA activity was recorded in treatment treated with 20 µg/mL of CC extract at 24h. Comparing to control, most of HKLs treated with extract fractions, except treatment of 40 µg/mL of EA, could significantly induced the RBA activity at 24h ($p<0.05$).

Also, the NOS activity was considerably increased in most of extract treatments at both time points ($p<0.05$). Only treatment stimulated with 10 µg/mL of CC statistically reduced the NOS activity at 6h ($p<0.05$) (Fig. 1B).

The lysozyme activity was early increased in HKLs treated with 40 µg/mL of CC and 10 µg/mL of EA at 6h, the level then continuously increased in most of extract treatments compared to control at 24h ($p<0.05$) (Fig. 1C).

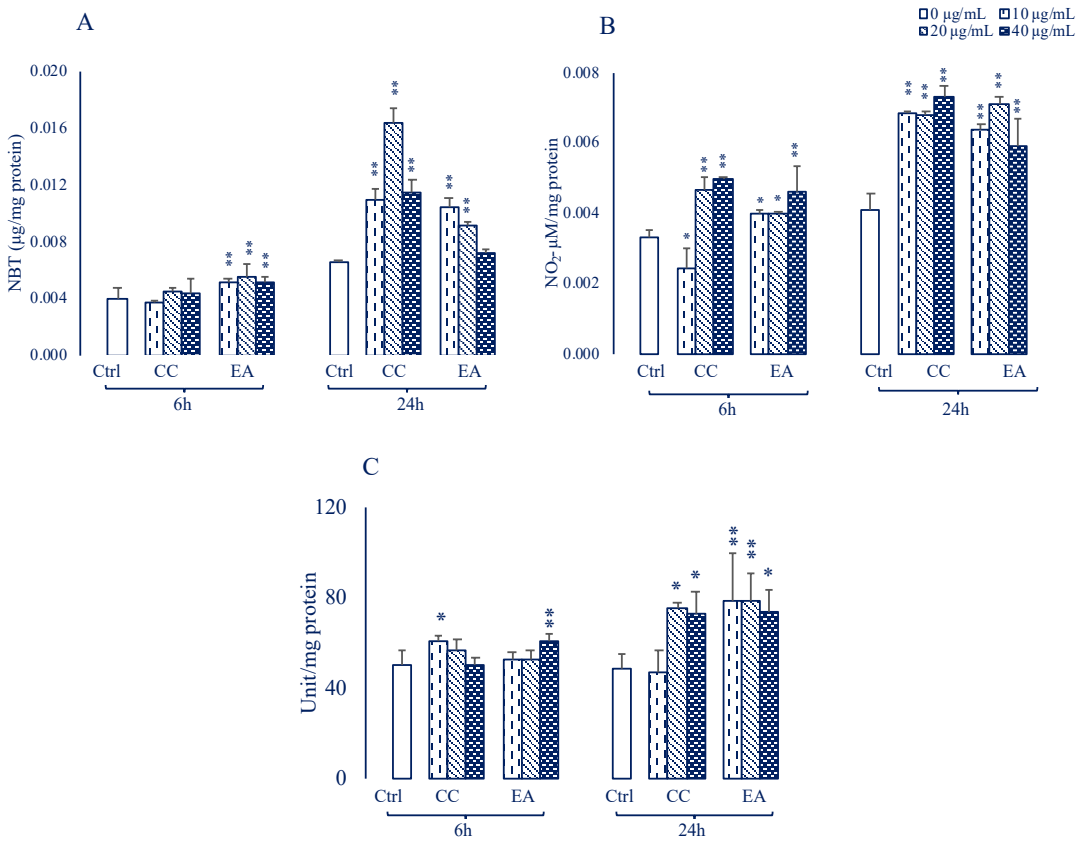

Figure 1. Immune parameters A) Respiratory burst activity- RBA, B) Nitric oxide synthase-NOS, and C) lysozyme of striped catfish head kidney leukocytes stimulated with different concentrations of each dichloromethane (CC) or ethyl acetae (EA) extract fraction. Values are presented as means ±SD.

3.2. Time and dose-dependent modulation of the RBA, NOS and lysozyme activities of striped catfish stimulated by CC and EA fractions

The results indicated that CC and EA fractions did not show any significant increase in spleen RBA, spleen NOS and serum lysozyme activities at 6h (Fig. 2). After 24h of injection, CC fraction at 40 µg/fish significantly inhibit the RBA and NOS activities in spleen, whereas EA fraction (10 µg/fish) could significantly increase the levels of RBA, NOS and lysozyme activities when compared to control ($p<0.05$). Fish treated with high dose of CC statistically enhanced the RBA and NOS activities in spleen at 72h ($p<0.05$). Similarly, the significant increase of the NOS level was also observed in fish treated with high dose (40 µg/fish) of EA extract fraction ($p<0.05$). Only treatment treated with EA fraction (10 µg/fish) could significantly increase the serum lysozyme activity ($p<0.05$) at 72h.

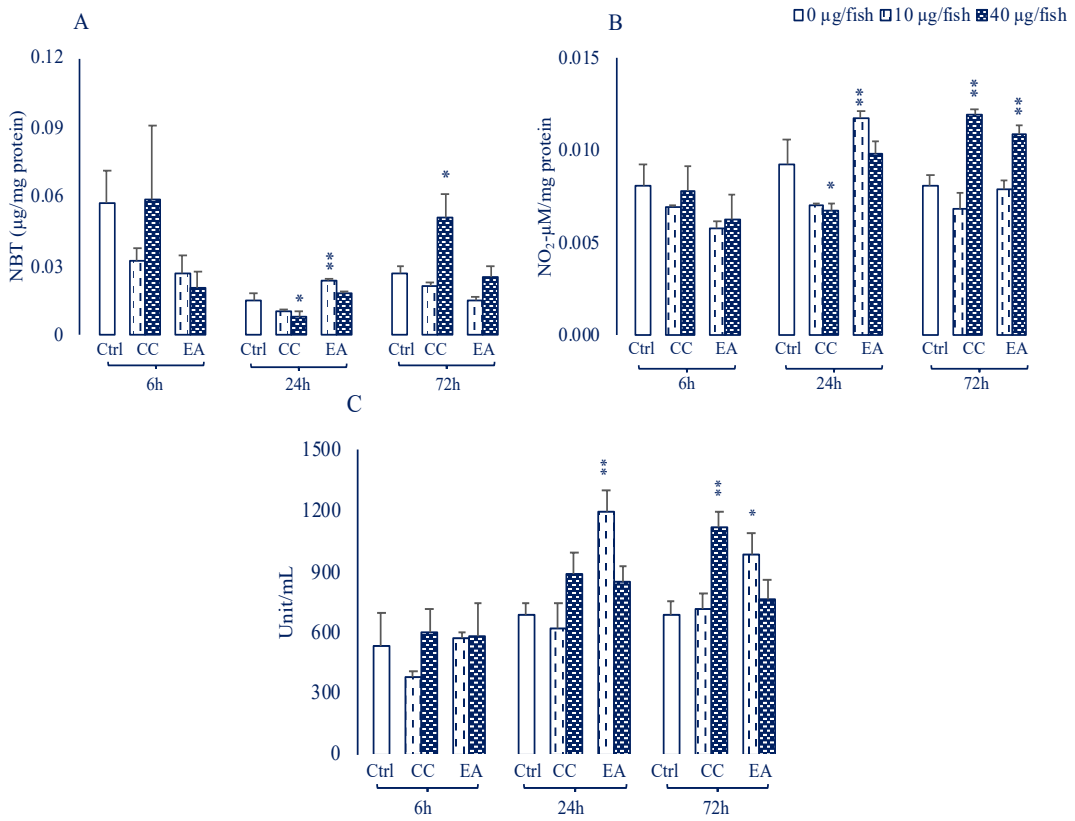

Figure 2. Immune parameters A) Respiratory burst activity- RBA, B) Nitric oxide synthase-NOS, and C) lysozyme of striped catfish were intraperitoneally injected with different concentrations of each dichloromethane (CC) or ethyl acetae (EA) extract fraction. Values are presented as means ±SEM.

3.3. Time and dose-dependent modulation of cytokine expression in head kidney of striped catfish treated with CC and EA fraction

3.3.1. Expression of cytokines involved in innate and adaptive immune response

After stimulated with extract fractions, the innate immune status of striped catfish was assessed by measuring the mRNA levels of *lys*, *inos*, *tlr1*, *tlr2*, *tlr4*, *myd88*, *traf6* and *mhc II* in head kidney at different time points (Fig. 3). *Lys* expression showed a significant variation following dose and sampling time. Both CC and EA fractions did not affect to the mRNA level of *lys* at 6h after injected. However, the *lys* expression was significantly upregulated in fish treated with 10 µg/fish of EA fraction at 24 ($p<0.05$). Such increments were also detected in high dose of CC fraction at 72h.

Fish treated with both doses of CC fraction resulted in a significant increase in the mRNA level of *myd88* compared to control at an early time- 6h ($p<0.05$), although *myd88* levels were not changed after treated with EA fraction. The *myd88* expressions were then returned to the basal level in most of treatments at 24 and 72h.

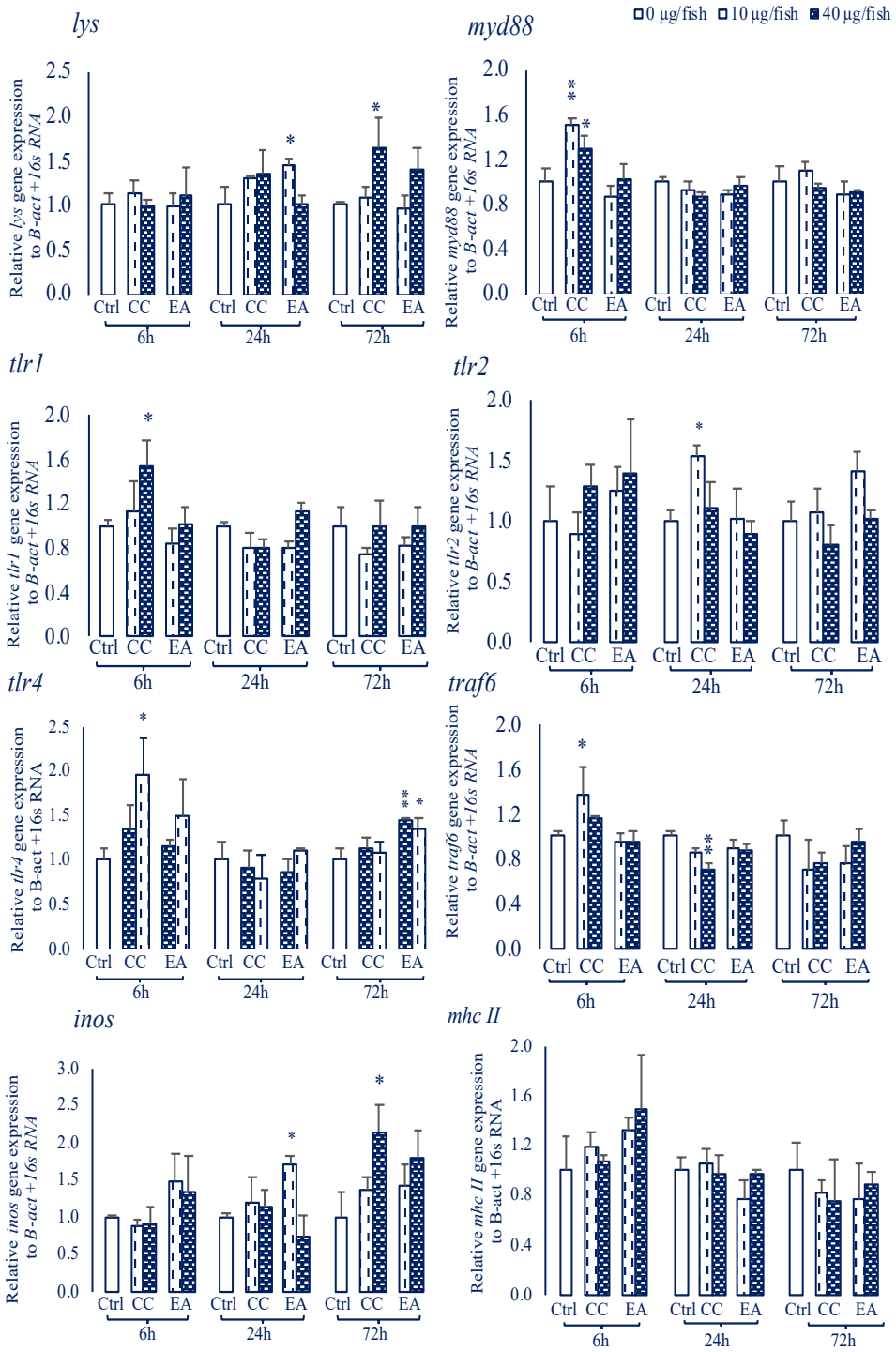

Figure 3. Expression of cytokine genes related to immune response in head kidney of striped catfish at 6, 24 and 72h after intraperitaneous injection of *P. guajava* extract fractions (40 and 10 µg per fish). Bars represent the mean ± SEM (n = 3) of relative mRNA expression as a fold change relative to beta actin and 16s RNA. Significant differences compare to control treatment: *p < 0.05, **p < 0.01. CC: Dichloromethane, EA: ethyl acetate.

Concerning mRNA expression of *tlrs*, the *tlr1* mRNA reached a maximum level in fish treated with high dose of CC fraction at 6h, and the levels were then returned to baseline at 24 and 72h post stimulated. The fish injected with the low dose of CC fraction only significantly increase the expression level of *tlr2* at 24h. Additionally, at the early time points, the mRNA level of *tlr4* in head kidney were significantly enhanced in fish treated with high dose of CC fraction. Moreover, an significant upregulation of *tlr4* mRNA level was also observed in fish treated with both dose of EA fraction at 72h, compared to control ($p<0.05$).

As regards to *traf6* expression, the maximum level was recorded in striped catfish treated with low dose of CC fraction at 6h compared to control ($p<0.05$). Conversely, the *traf6* expression was significantly downregulated in fish treated with high dose of CC at 24h($p<0.01$). No significant differences in *traf6* expression was observed in other of experimental treatments compared to control treatment at 72h ($p>0.05$).

In the early time-6h of stimulation, the mRNA level of *inos* was not affected by extract fractions ($p>0.05$). While significant higher expression levels ($p<0.05$) of *inos* were detected in fish treated with low dose of EA fractions at 24h. The *inos* level was significantly increased in high dose of CC fraction compared to control at 72h ($p>0.05$).

Both doses of CC and EA fractions did not significantly enhance the mRNA expression of *mhc class II* throughout the sampling times.

3.3.2. Expression of cytokines related to inflammatory response

The levels of inflammatory cytokines displayed a wide variation in response according to the kind of fraction used (Fig. 4). Fish treated with CC and EA fractions did not affect to the mRNA expression of *nfκb1* at all time points. However, an early upregulation (at 6h) of *nfκb2* expression was observed in fish treated with 10 µg/fish of CC compared to control ($p<0.01$), the level was returned to the baseline at the later time points. Although the level of *nfκb2* was not affected by EA fraction stimulation at 6h, its expression at the low dose was started to significantly increase at 24h compared to control and gradually remained until 72h post stimulation.

Concerning mRNA levels of *il1β* and *il6*, they both showed an statistical increase at 6h post treated with CC fractions compared to control treatment ($p<0.01$), and then returned to normal immune homeostasis at 24h. Similarly, fish treated with high dose of CC fraction also significantly enhanced the *tnf* in head kidney ($p<0.05$). However, no differences in the *il1β, il6* and *tnf* expression were detected in fish treated with EA fraction throughout the sampling time points ($p>0.05$).

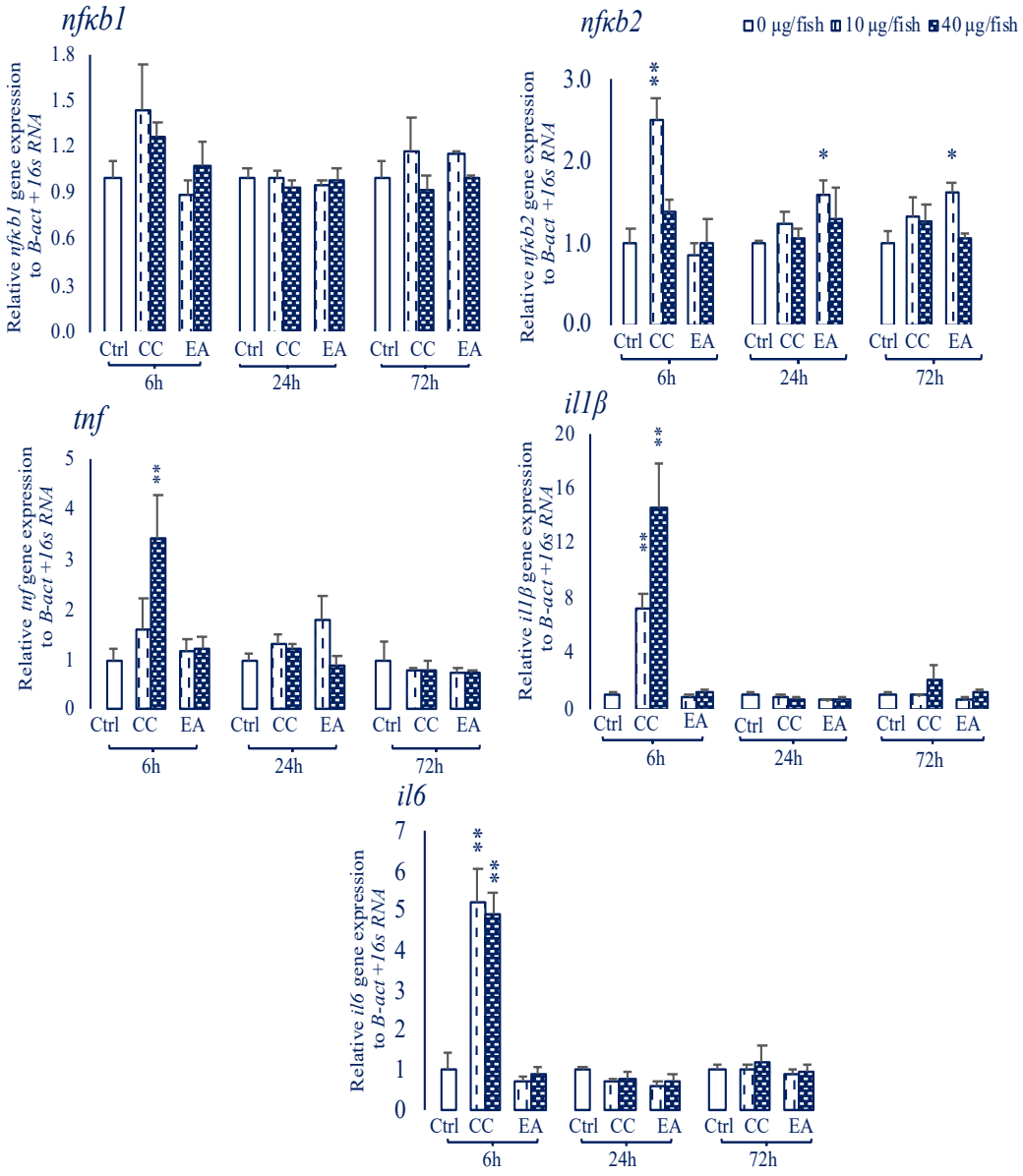

Figure 4. Expression of cytokine genes related to inflammatory in head kidney of striped catfish at 6, 24 and 72h after intraperitaneous injection of *P. guajava* extract fractions (40 and 10 µg per fish). Bars represent the mean ± SEM (n = 3) of relative mRNA expression as a fold change relative to beta actin and 16s RNA. Significant differences compare to control treatment: *p < 0.05, **p < 0.01. CC: Dichloromethane, EA: ethyl acetate.

3.3.3. Expression of cytokines related to apoptosis process

In striped catfish head kidney, the relative expression of *casp3* were not influenced by CC or EA treated during the sampling times (p>0.05) (Fig. 5). While significant upregulation in *casp8* level were observed in both doses of CC fraction at 6h post stimulation (p<0.05). Similar observations was made in low dose of CC and both doses of EA fractions at 24h (p<0.05). Furthermore, treating fish with both doses of CC fraction could cause a significant decrease of *casp8* expression at 72h compared to control (p<0.05). Additionally, only fish treated with low dose of CC fraction significantly upregulated *tp53* expression at 6h (p<0.05).

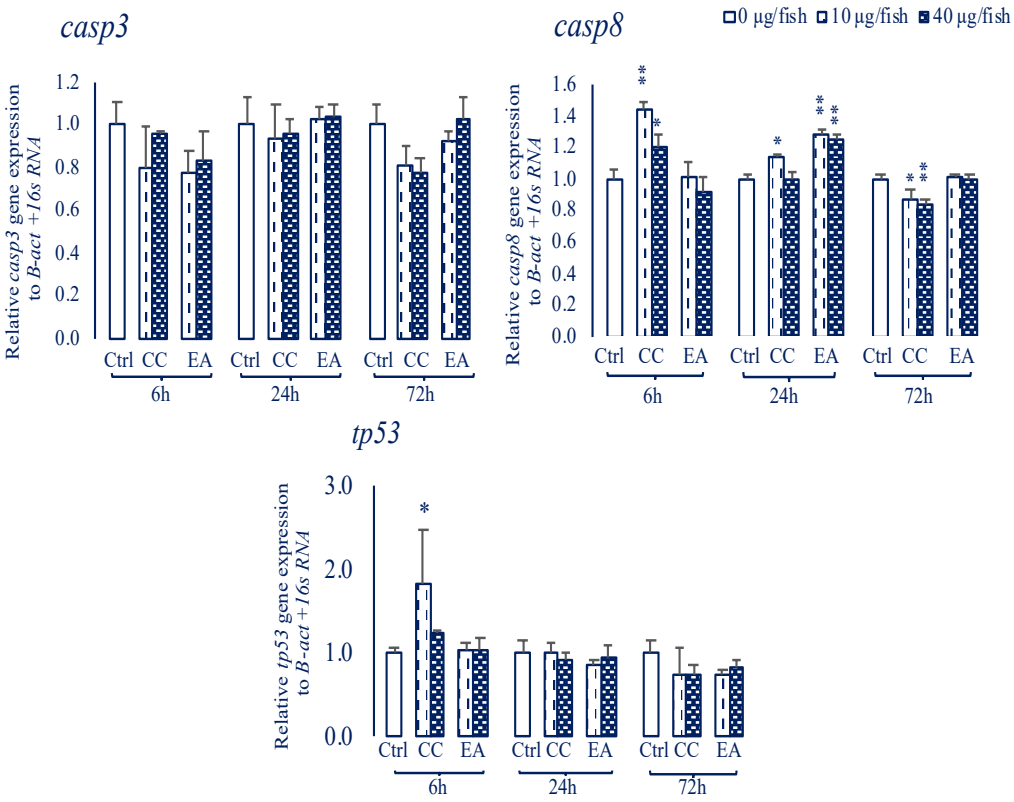

Figure 5. Expression of cytokine genes related to immune response in head kidney of striped catfish at 6, 24 and 72h after intraperitaneous injection of *P. guajava* extract fractions (40 and 10 µg per fish). Bars represent the mean ± SEM (n = 3) of relative mRNA expression as a fold change relative to beta actin and 16s RNA. Significant differences compare to control treatment: *p < 0.05, **p < 0.01. CC: Dichloromethane, EA: ethyl acetate.

4. Discussion

The present study aimed to highlight the early effects of different *P. guajava* extract fractions by looking for changing levels on inflammation as well as immune response of striped catfish after intraperitoneal administration at different time points, followed by assessing their underlying mechanisms. Plant products have been identified as potential therapeutic treatments by modulating immunity as well as by preventing or controlling fish diseases (Van Hai, 2015, Awad and Awaad, 2017, Galina *et al.*, 2009). Different polarities of the extraction solvents may cause a wide variation in the level of bioactive compounds in the extract, resulting in various extraction yields (Truong *et al.*, 2019). In the previous *in vitro* study, crude ethanol extract from *P. guajava* was fractionated by different solvents including n-hexane, dichloromethane, ethyl acetate, and water. The products from dichloromethane and ethyl acetate, which strongly enhanced the immune response in striped catfish head kidney leukocytes, were intraperitoneally injected to striped catfish to access for homeostasis mechanism. In the previous study (Chapter 7), the phytochemical constituents of crude ethanol extract of *P. guajava* and its fractions were investigated. The major components identified in crude *P. guajava* were flavonoids and triterpenics, whereas its dichloromethane fraction mainly

contained triterpenic derivatives and its ethyl acetate fraction contained both phenolic and triperpenic derivatives. Moreover, the dichloromethane and ethyl acetate fractions of *P. guajava* possessed the better immune responses in striped catfish HKLs compared to other fractions (aqueous and *n*-hexane fractions).

Recently, natural plants have attracted considerable attention as a valuable source of nutritional ecology used in aquaculture industry (Awad and Awaad, 2017, Chakraborty and Hancz, 2011). For this reason, understanding the functional mechanisms of bio-products is an important step forward to improve fish health as well as prevent pathogen infection, morbidity and mortality in aquaculture setting. In the present study, *in vitro* and *in vivo* tests indicated that the CC and EA extract fractions acted as immunostimulators via their capacity in enhancing RBA, NOS and lysozyme activities. Reactive oxygen species-ROS primarily appear during phagocytosis, which is mainly released on the endosomal membranes of the phagocytosing cells (i.e. neutrophils and macrophages) (Grayfer *et al.*, 2018). RBA is the rapid release of reactive oxygen species from those phagocytosis cells (Srivastava and Pandey, 2015). Nitric oxide is an important mediator of biological and immune functions, including inflammation and cytotoxicity to invading organisms. Moreover, the nitric oxide response is subsequent and links together with the respiratory burst by a tryptophan degradation responses (Atakisi and Merhan, 2017). We currently observed that the RBA activity significantly increased in an early time point-6h in HKLs stimulated with EA fraction, whereas the RBA level in CC treatments could significantly increase at the later time point- 24h post stimulation. Conversely, HKLs stimulated with most of extract fractions significantly enhanced the NOS activity at 6 and 24h. However, intraperitoneally administration of fish by both extract fractions did not affect to the RBA level at 6h. The upregulation or downregulation of RBA and NOS activities differed depending on time and dose. In line with our results, Mbaveng et al. (2018) also indicated that guava bark extract significantly increased the production of ROS in leukemia CCRF-CEM cells at 24h (Mbaveng *et al.*, 2018). A document by Nguyen et al. (2019) demonstrated that guava leaves extract also significantly enhanced the ROS activity in the human hepatocellular carcinoma cell line HepG2 at 48h (Nguyen *et al.*, 2019). Moreover, ethyl acetate extract from murtilla- *Ugni molinae* Turcz increased the nitric oxide availability in bovine aortic endothelial cells via activation of protein kinase B (AKT) and eNOS phosphorylation (Arancibia-Radich *et al.*, 2019). Additionally, we also found that CC fraction significantly inhibited the release of ROS and NOS activities, whereas EA fraction significantly enhanced these activities at 24h. It could be concluded that biological activities related to immune responses may be strongly affected by the kind of extract solvent.

Belonging to the humoral immune response and mainly produced in neutrophils (Fletcher and White, 1973), lysozyme plays an important role in host mediating protection against microbial invasion. In the present experiment, both CC and EA fractions significantly increased the lysozyme activity in the *in vitro* testing, whereas only low dose of EA fraction could significantly stimulate the lysozyme level in the *in vivo* experiment. As mentioned above, activating the macrophages not only led to the release of RBA and NOS activities but also increased the lysozyme activity (Yu *et al.*, 2013). However, the lysozyme results in the present

study not always corresponded to the RBA or NOS activity discussed earlier. This may be due to the fact that *P. guajava* extract possesses some flavoinoid compounds, which strongly bind to the number and position of hydrogen and glycosides of lysozyme, then inhibited the lysozyme activity (Yang *et al*., 2012). Moreover, some exhibited degrees of ROS, NOS and lysozyme activities could also be due to the abundance flavonoid and triterpenic derivatives in the *P. guajava* fractions. In the previous study (Chapter 7), triterpenic derivatives including (corosolic, oleanolic, and ursolic acids), which were the major components of both CC and EA fractions, positively enhanced the RBA and lysozyme activity. In addition, flavonoid derivatives (i.e. dihydroquercetin, quercetin, and kaempferol) could also increase the phagocytosis, RBA, and lysozyme levels in several fish species (Awad *et al.*, 2013, Awad *et al.*, 2015, Chi *et al.*, 2016).

Aside from the humoral and cellular immune responses, our study also provided evidence that both guava extract fractions could positively regulate the cytokine -related gene expressions in striped catfish at the early time of stimulation. Indeed, relative *inos* and *lys* expressions were differentially enhanced in dose and time dependent manners. The increase level of *inos* and *lys* was accompanied by NOS and lysozyme activities in the spleen and serum of striped catfish, respectively. Like mammals, fish recognized pathogens via various types of cell surface pattern recognition receptors, such as toll like receptors (TLRs) (Takeda and Akira, 2005). Our study highlighted that mRNA levels of *tlr1* and *tlr4* were significantly increased at high dose of CC group ($p<0.05$), although the *tlr2* expression was slightly increased in fish treated with both CC and EA fractions ($p>0.05$). In agreement with our results, Chen et al. (2017) also reported that polysaccharide in the *Salvia miltiorrhiza* Bunge water extract significantly enhanced transcript levels of *tlr1*, *tlr2* and *tlr4* in peripheral blood T lymphocytes of cancer patients (Chen *et al.*, 2017). It was indicated that MyD88 played a crucial role in innate immunity signal transduction (Warner and Núñez, 2013). In the present study, the *myd88* expression was found to be significantly upregulated in fish treated with both doses of CC fraction at 6h, and then the *myd88* transcript was reduced to the basal immune homeostasis. In line with our results, Su et al., (2011) also demonstrated that mRNA level of *myd88* in grass carp (*Ctenopharyngodon idella*) head kidney cells treated with polycytidylic acid were considerably increased at early time (2 and 8h), and then returned to the control level at 24h (Su *et al.*, 2011). Similar increment of *myd88* expression were also observed in grass carp head kidney macrophages stimulated with kaempferol 3-a-L-(4-O-acetyl) rhamnopyranoside-7-a-L-rhamnopyranoside compound at 8h (Chi *et al.*, 2016). TLR signaling pathways are mediated by interactions between MyD88 and TLRs, then TRAF6 is activated (Nie *et al.*, 2018). We currently also find that the CC and EA fractions could regulate the *traf6* expression depending on time and dose. Arain et al. (2019) indicated that the mRNA level of *traf6* was significantly upregulated in goat peripheral blood leukocytes stimulated with *Debaryomyces hansenii* β-glucan (Arain *et al.*, 2019). However, our results also indicated that the mRNA expression level of *Mhc class II* was not affected by in fish treated with CC and EA fractions, suggested that *P. guajava* fractions may not stimulate the adaptive immune response in the early time of treatment.

Activating TLRs signaling pathways following recruitment MyD88 and TRAF6 adaptor proteins results in the production of proinflammatory cytokines, and adaptive immunity (Su *et al.*, 2011). Among the inflammatory cytokines, the nuclear transcription factor NFκB has been considered in the regulation of various gene expressions that was vital in apoptosis and immunomodulation (Lawrence, 2009). In the current study, the *nfκb2* transcript level was significantly upregulated at an early time point -6h in fish treated with CC fraction, whereas the low dose of EA treatment could significantly stimulate the *nfκb2* expression at the later time point- 24h and the level increased until 72h. RualaCap, a bioactive fraction isolated from *Russula alatoreticula* potentially enhanced the transcript level of *nfκb* in RAW 264.7 murine macrophages (Khatua and Acharya, 2019). Also studying RAW 264.7 macrophages, Shen et al (2017) suggested that polysaccharides isolated from wheat bran rapidly upregulated mRNA level of *nfκb* via *tlr4* activation (Shen *et al.*, 2017). Aside from the NFκB cytokine, TNF- α, IL-1β and IL-6 are vital keys in inflammatory response (Wojdasiewicz *et al.*, 2014). Moreover, the increase of IL-1β could stimulate the production of ROS (Afonso *et al.*, 2007), while TNF-α triggered lysozyme production by macrophages (Ogundele, 1998). Additionally, IL-1β and TNFα present together could enhance the production of iNOS (El Mansouri *et al.*, 2011) and notably they regulate the expression of IL-6 (Palmqvist *et al.*, 2008). Interestingly, our results found that the mRNA levels of *tnf*, *il1β* and *il6* were considerably upregulated and seemly consisted together in fish treated with CC fraction at 6h. Similar increment of *il1β* transcript was also observed in common carp (*Cyprinus carpio*) fingerlings fed with diets enriched with guava leaf powder (Hoseinifar *et al.*, 2019b). A study of Giri et al., (2015) observed that 0.5% guava leaves extract- based diets could upregulate levels of *il-1β* and tumor necrosis factor alpha (*tnfα*), whereas downregulate *inos*, *nfkb*, cyclooxygenase-2 (*cox2*), and transforming growth factor beta (*tgfβ*) expression levels in different tissues of rohu (Giri *et al.*, 2015). However, using high concentration of guava leaves could significantly inhibit the expression level of iNOS and cyclooxygenase-2 protein levels via the down-regulation of NF- κB in mouse macrophage cells after inflammation stimulated by LPS (Choi *et al.*, 2008). Flavonoid fraction of guava leaf extract could reduce the NF-κB activation in rohu *Labeo rohita* head-kidney macrophages via LPS stimulation (Sen *et al.*, 2015). Taken together, these studies combined with our results confirmed the expected role of guava extract as not only activating the proinflammatory cytokines during onset of inflammation, but also enhancing the anti-inflammatory cytokines during the resolution of inflammation. This outcome could suggested that further studies should be undertaken about the optimize dosages to be employed the best immunotherapeutic applications of guava extracts.

Concerning the apoptosis process, our results showed that *casp3* transcript level were not significantly affected by both extract fractions. However, CC and EA fractions could differentially regulate the expression of *casp8* in head kidney of striped catfish. The same results were reported by Mbaveng et al (2018) when CCRF-CEM cells stimulated with *P. guajava* bark extract could activate the CASP3/7, 8 and 9 activities, and then results in inducing apoptosis (Mbaveng *et al.*, 2018). A significant increase of CASP8 and CASP9 activities was also observed in SW480 cells stimulated with *Teucrum chamaedrys* L. extract (Milutinović *et*

al., 2019). CASP8 activation occurs via both the death receptor pathway and the mitochondrial pathway. Moreover, P53 family-including *tp53* transcript is an important upstream regulator of caspase activation (Liu *et al*., 2011). In the present study, only a low dose of CC fraction significantly enhanced the mRNA level of *tp53* in head kidney of striped catfish at 6h, and then the expression reduced to the basal level at 24h.

Generally, we identified traits associated with two different *P. guajava* fractions (CC and EA)-stimulated striped catfish in the early stage. It was well documented that TLRs and MyD88 are upstream regulatory factors of NF-κB (Afonso *et al.*, 2007). In the present results, the TLRs-MyD88-NF-κB signaling pathways were mostly enriched in the CC group. Belong to the pattern recognition receptors (PRRs), TRLs are responsible for the most extensive spectrum of pathogen recognition including the pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) (Nie *et al.*, 2018). Our results showed that *tlr1* and *tlr4* were firstly activated, followed by the upregulation of *myd88* at 6 h in CC group. Upon PAMPs and DAMPs recognition, TLRs recruit MyD88, which initiate signal transduction pathways that culminate in the activation of NF-κB, to regulate the expression of cytokines, chemokines, and type I IFNs that ultimately protect the host from microbial infection (Kawasaki and Kawai, 2014). The present results indicated the activation of TLRs/MyD88 was involved in the production of inflammatory cytokines in striped catfish head kidney including *nfκb2*, *tnf*, *il1β* and *il6*. This trend was also observed in the subsequent detection of cell apoptosis and the identification of apoptotic pathway genes (*casp8* and *tp53*). The activation of TLRs signaling pathway also stimulated to increase the production of mitochondrial ROS for bactericidal action and recruit mitochondria to phagosomes (West *et al.*, 2010). The increment of RBA and NOS production was also indicated in our results. These results indicated that the CC positively affected to the striped catfish immune responses.

5. Conclusions

Our study highlighted that the intraperitoneal injection of CC and EA fractions positively enhanced humoral immune response (lysozyme) and cellular immune response (NOS and ROS) in striped catfish in both *in vivo* and *in vitro* experiments. Moreover, these extract fractions, especially CC fractions, potentially upregulated the expression of cytokine genes involved in immune response, inflammatory and apoptosis at an early stage. The TLRs-MyD88-NF-κB signaling pathway was activated, following an induce of the production of inflammatory (*nfκb, tnf*, *il1β* and *il6*), apoptosis (*tp53* and *casp8*), lysozyme, iNOS and ROS. These functions could possibly be attributed by some active compounds present in the *P. guajava* fractions. The results supported the possibility of using *P. guajava* as a potential natural pharmaceutical in fish. However, additional studies are still needed to determine the mechanisms by which the active compounds present in this plant could induce the immunomodulation.

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SECTION III

Chapter 9

General discussion, conclusions, and perspectives

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1. General discussion

World aquaculture production has been revealed as the main source for human food safety and nutritional demands worldwide. Among the aquatic animals, the striped catfish (*Pangasianodon hypophthalmus*) has become an economically important fish and rapidly grown in several Asian countries. In parallel with the development, the striped catfish sector has been reported to occur with huge economic losses and reduced profit margins because of many reasons. One of the major problems is the outbreak of infectious diseases (i.e. bacteria, fungi, and parasites) which frequently come from the stress factors resulting from the intensification aquaculture practices. As the demand for an increase in food quality, the side effects of antibiotics become an increasing threat to humans and environment. Therefore, the solution should be to eliminate as much as possible the use of antibiotics and to minimize them by alternative developed products. Nowadays, the most efficient methods in reducing mortality involve strengthening fish defense mechanisms. From this perspective, natural plant-derived bio-products have attracted considerable attention as a source of eco-friendly prophylactic compounds in the aquaculture industry. Many medicinal plant products have been demonstrated to successfully improve immune responses and disease resistance in several fish species. However, these products are not yet popularly applied in striped catfish culture. Farmers lack knowledge regarding the existence of such bio-active products or their efficacy in fish.

The main objectives of this thesis were, from a fundamental perspective, to get a better understanding of the immunomodulatory mechanisms of plant extract administration in striped catfish via *in vitro* and *in vivo* approaches, and, from an applied perspective, to determine the optimal doses, and duration of treatment of several plant extracts, as well as their efficiency on striped catfish immune responses and resistance to a bacterial infection (*Edwardsiella ictaluri*). Indeed, an *in vitro* screening of 20 herbal plant extracts was conducted for determining their effects on immune responses of striped catfish leukocytes (Chapter 4). Five ethanol extracts

selected from the screening test including *P. amarus*, *P. guajava*, *M. pudica*, *A. indica*, and *E. hirta* were validated via *in vivo* experiment. These extracts were then supplemented to diet of striped catfish for 8 weeks and we evaluated the effects on blood parameters, immune responses and bacterial resistance in striped catfish against infection with the *E. ictaluri* pathogen (Chapter 5). Moreover, the synergistic effects of single supply compared to the mixture supply of *P. amarus* and *P. guajava* extracts on immune responses, disease resistance and liver proteome profiles in striped catfish were investigated after 6 weeks of plant extract feeding (Chapter 6). In addition, to obtain more information about the potential active compounds from the crude ethanol extracts, we fractionated extracts with solvents of different polarities to separate the classic components which may result in various immune functions in fish. The immunomodulatory effects of crude ethanol extract of *P. amarus* and *P. guajava*, their fractions including n-hexane, dichloromethane, ethyl acetate, water and non-tannin, as well as pure compounds, were compared, *in vitro* using striped catfish head kidney leukocyte model (Chapter 7). Finally, the mechanism of two *P. guajava* extract fractions (dichloromethane and ethyl acetate) on striped catfish immune responses was assessed using *in vitro* and *in vivo* dynamic models (Chapter 8).

1.1. Effects of plant extract on striped catfish immune responses

Effects of plant extracts and fractions on PBMCs and HKLs

Twenty ethanol plant extracts, which are popularly distributed in the Mekong Delta of Vietnam, were demonstrated to possess the immunomodulatory activities in striped catfish leukocytes (PBMCs and HKLs) via activating the humoral immune responses (i.e. lysozyme, complement, and total immunoglobulin) (Chapter 4). Despite using the same method, differences were noticed in the activity levels of lysozyme, complement, and total immunoglobulins in cells after stimulated with different plant extracts. Furthermore, the humoral immune responses were also variably depending on the extract concentration and type of stimulated cells. In this study, five extracts including *A. sativum* , *A. indica*, *E. hirta*, *P. amarus*, and *Z. officinale* were efficient in stimulating the humoral immune responses as well as the expression of 4 cytokines (i.e. *il1β*, *ifnγ* 2a and 2b, and a2 *mhc class II*) after 24h. In contrast, *C. asiatica, H. cordata, M. pudica* extracts were less effective on the immune parameters of striped catfish leukocytes. However, our study did not find any inhibited effects of the extracts at the examined concentrations (10 and 100 µg/mL) on the humoral immune response of striped catfish leukocytes.

Ethanol extracts exerted immunomodulatory effects via enhancing the humoral immune parameters in striped catfish leukocytes. Furthermore, several fractions of *P. guajava* (n-hexane, dichloromethane, ethyl acetate, aqueous) and *P. amarus* (n-hexane, ethyl acetate, aqueous, and non-tannin fractions) in a concentration dependent manner showed different regulation to the respiratory burst, nitric oxide synthase, and lysozyme activities (Chapter 7). Among the fractions, the dichloromethane and ethyl acetate fractions of *P. guajava* were better in stimulating these immune markers in HKLs. The variable levels could be due to the different constituents presented in each fractions (i.e. triterpenic and flavonoid derivatives were isolated

in *P. guajava* ethyl acetate fraction, while only triterpenic derivatives presented in its dichloromethane fraction) (Chapter 7). It could be suggested that the same plant origin and different extract solvents could yield different phytochemical constituents in each fraction and result in different HKLs immune responses. Concerning pure compounds, avicularin, guajaverin, corosolic acid, ursolic acid, olenanolic acid and hypophyllanthin significantly stimulated the ROS production as well as the lysozyme activity in HKLs in a dose dependent manner (Chapter 7). Several studies demonstrated that the ROS and NOS productions were significantly enhanced in infected macrophages stimulated with oleanolic acid and ursolic acid (López-García *et al.*, 2015, Ikeda *et al.*, 2007, Choi *et al.*, 2001). However, the NOS production was significantly reduced in HKLs treated with olenanolic acid (30 μM) and hypophyllanthin (7.5 μM).

Effects of plant extract on striped catfish immune responses

In the following chapter (Chapter 5), we evaluated the effects of 5 ethanol plant extracts (*P. amarus*, *P. guajava*, *M. pudica*, *A. indica* and *E. hirta*) during 8 weeks of oral administration on the immune responses of striped catfish. Plant extracts differentially improved the blood indices (white blood cells, monocytes, lymphocytes, and neutrophils), humoral immune response (lysozyme, complement, and total Ig) in serum and skin mucus of striped catfish. Nevertheless, results differ because the plants under study were different, also the duration of the treatment and the dose assayed. Regarding *M. pudica* extract, the results also addressed that the *in vitro* and *in vivo* results were not always consisted when compared together. *M. pudica* extract was less effects to the humoral immune response (lysozyme, complement, and total Ig) in striped catfish leukocytes (Chapter 4), whereas it strongly stimulated to enhance the serum and skin mucus immune parameters at the dose of 2% after 8 weeks of feeding (Chapter 5). The differences may be due to the kind of cell type (primary versus immortal) and methods of extract delivery (exogenous addition versus diet) (Rockett *et al.*, 2012). In addition, most of the plants were selected from different parts, such as *P. amarus* (leaves, twigs), *P. guajava* (leaves), *M. pudica* (leaves, twig), *A. indica* (leaves) and *E. hirta* (leaves, twigs), which could present of various constituents (Altemimi *et al.*, 2017), and result in different effects to the striped catfish immune responses. However, our thesis lacks of the study to compare the bioactive constituents as well as their immune responses on striped catfish between the different parts of plant.

Among the plant extracts examined, *P. guajava* extracts (both doses) outstandingly appeared as good immunostimulant candidate for boosting the striped catfish immune responses. Another study also demonstrated *P. amarus* could act as good antioxidant agent (data not show). The following study (Chapter 6) observed the optimized dose of single or mixture supply of *P. guajava* and *P. amarus* extracts in striped catfish. The extract of *P. guajava* and *P. amarus* alone or in combination positively regulated the cellular immune responses (RBA and NOS productions) in spleen and humoral immune responses (lysozyme, complement and total Ig) in skin mucus and plasma, although the mixture supply did not show any additive effect on the striped catfish immune responses (humoral and cellular) as compared to single supply.

Furthermore, seasonal changes could affect the quantity of phytochemical constituents of plant extracts (Gololo *et al.*, 2016). Regarding *P. guajava* and *P. amarus* extracts, the fresh plants were collected at two seasons from October to November of 2016 (Chapter 5), and from April to May of 2017 (Chapter 6). However, two studies (Chapter 5 and Chapter 6) did not show the different effects between *P. guajava* and *P. amarus* extracts. These extracts at low dose significantly enhanced the immune responses of striped catfish after oral administration in both experiments.

1.2. Effects of oral administration of plant extracts on striped catfish mortality after *Edwardsiella ictaluri* infection

Bacillary Necrosis Pangasius caused by *E. ictaluri* frequently occurs and is currently the most economically serious disease of intensively reared striped catfish (Ferguson *et al.*, 2001, Crumlish *et al.*, 2002). Striped catfish fed diets supplemented with five ethanol extracts (*P. amarus*, *P. guajava*, *M. pudica*, *A. indica* and *E. hirta*) dose-dependently reduced the cumulative mortality after challenge test with *E. ictaluri.* The mortality started to occur in most of the treatments from day 4 post-infection (Chapter 5). Strengthening the fish health status could result in the better protection of striped catfish against the *E. ictaluri* infection. 0.2 and 1% of *P. guajava* extract-based diets not only showed the optimal potential for modulating blood parameters and immune responses (humoral and mucosal) in striped catfish but also reduced the mortality after bacterial challenge test (Chapter 5). In the contrary, beside playing an important role in immunomodulation in host, plant extracts also function as antibacterial agent (Harikrishnan *et al.*, 2011, Van Hai, 2015, Awad and Awaad, 2017). Of the different plant extract diets, 0.4 and 2 % of *M. pudica* extract-based diets displayed the highest survival rate post-challenge, although *M. pudica* extract did not outstandingly show significant effects on striped catfish PBMCs and HKLs in the *in vitro* test. The low dose of *M. pudica* may not stimulate better the immune responses but helped to reduce the losses caused by diseases. It could be due to *M. pudica* could possess antibacterial activity. Moreover, there was no positive correlation between the concentrations and dosages on the fish immune responses (Harikrishnan *et al.*, 2011). The ethanol extracts including *P. amarus* and *A. indica* significantly reduced the mortality at the low dose (0.2 and 0.4%, respectively), whereas these extract at the high doses (1 and 2%, respectively) did not significantly protect the striped catfish after challenge test.

In addition, single *P. amarus* or *P. guajava* extract-based diets did not significantly reduce the striped catfish mortality at the lowest dose (0.08%), whereas their mixture at the same dose was recorded with the significantly lower mortality rate compared to control (Chapter 6). It could be suggested that the combination of *P. amarus* and *P. guajava* extracts resulted in an additive effect on fish capacity against *E. ictaluri* infection. Moreover, the higher doses (0.2 and 0.5%) of *P. amarus* and *P. guajava* in combination or single extract (except 0.2% of mixture) also significantly reduced the mortality in striped catfish. These evidences demonstrated that *P. amarus* and *P. guajava* showed the best protection of striped catfish against bacterial infection after oral administration.

1.3. Dose dependent manners of plant extract administration

In chapter 5, most of the immune parameters increased in several treatments after 4 weeks of feeding. Moreover, the significant increase was clearly displayed after a long term feeding-8 weeks, which showed better protection in fish against *E. ictaluri* bacteria. Indeed, the low dose of *P. amarus* (0.2%), high dose of *M. pudica* and *E. hirta* evidently enhanced the blood indices as well as humoral immune responses at week 8 and day 3 post-challenge. Most of the immune parameters significantly increased in *P. guajava* (0.2 and 1%) treatments at week 4, week 8 and day 3 post-challenge. In contrast, the high dose of *P. amarus*, the low dose of *M. pudica* and *E. hirta*, and both doses of *A. indica* were occasionally found to be less effective to the striped catfish immune responses throughout the sampling times. The absence of a linear relationship between dose and effect was also documented by other authors for other plants and other species of fish (Xie *et al.*, 2008, Harikrishnan *et al.*, 2009, Kirubakaran *et al.*, 2010, Mohamad and Abasali, 2010, Soltani *et al.*, 2010). The variety of doses depends on several factors including herbal and fish species, as well as application methods. The practical dosages are generally small amount of herbal medicines (Van Hai, 2015). However, the time of administration incorporated with dosage not independently (Awad and Awaad, 2017). Additionally, the low doses of *P. guajava* and *P. amarus* at 0.08, 0.2 and 0.5% were investigated when striped catfish fed supplemented diets in a single or combination supply for 6 weeks (Chapter 6). The results showed a progressive increase in the immune capacity of striped catfish fed with an increased level of single extract diets, although their lowest doses (0.08%) did not significantly induce immune-stimulation. However, the mixture diets at the lowest dose (0.08%) significantly boosted the striped catfish immune responses at week 3, week 6, and more clearly at day 3 post-infection. In our study, there was not expected for any toxic doses among the extracts throughout the experiments (Chapter 5 and Chapter 6). An overdose of plant extracts did not significantly suppress or improve the immune responses as well as the capacity against bacterial infection. In agreement with our results, oral administration of aqueous *P. guajava* extract at 100–500 mg/kg body weight was relatively safe in Wistar rats up to 72 h (Etuk and Francis, 2003). In addition, an acute toxicity study of ethanol extract of guava leaves showed no signs of toxicity or cause mortality in albino rats even at doses >2000 mg/kgbody weight (Dutta and Das, 2010). However, more experiments need to be conducted for toxicity evaluation of plant extracts on striped catfish before applying them in striped catfish commercial culture.

The different effects on fish health status (humoral and cellular immune mechanism) after *P. guajava* and *P. amarus* extract-based diets were also detected depending on the periods of feeding trials. (Gobi *et al.*, 2016) showed that *P. guajava* ethanol extract at the optimal dose (1%) enhanced the health status of tilapia after 30 days post-feeding, whereas the best immune responses in rohu were observed after the longer term- 60 days with the lower dose (0.5%) of *P. guajava* ethanol extract-based diets. Our study highlighted that both hematological parameters and humoral immune responses of striped catfish were differently improved according to the dose and type of extract, as well as to the duration of feeding.

1.4. Effects of extract based-diets on liver proteome profiles

The medicinal plants have been demonstrated to positively improve the immune responses and disease resistance in aquatic animals, although the understanding of their mechanism of action has been limited. Proteomic analysis provides trusted information about the protein texture of healthy fish and/or secretions. It gives new insights on the biological state of fish in response to wide range of stimulants or diseases. In the present study, a proteomic approach was used in order to deeply elucidate the mechanism underlying immune as well as antioxidant tolerance of striped catfish liver in responses to oral administration of plant extracts (Chapter 6). The study suggested that the striped catfish liver proteome can respond quite differently to single or combination supply *P. guajava* and *P. amarus* extracts. It means that the expression levels of proteins related to the tested pathways were differentially regulated according to both the extract-type and the concentration after a long term of feeding. Seven pathways related to the immune and antioxidant processes were enriched in striped catfish liver following extract-based diets. Among the pathways, the proteins involved in the leukocyte transendothelial migration pathway were increased in most of the extract treatments, except the dietary treatment enriched with 0.08% *P. amarus*. Most of proteins related to antigen processing and presentation, natural killer cell mediated cytotoxicity, calcium signalling pathway, and NOD-like receptor signalling pathway were downregulated in the single supply of *P. amarus* or *P. guajava* extracts, while they were upregulated under a mixture of plant extracts. Of the proteins examined, CASP8 which is predicted to belong to both NOD-like receptor signalling and apoptosis pathways, significantly increased in all extract treatments. Two proteins including CLDN4-belonged to the leukocyte transendothelial migration pathway and NLRP12-belonged to NOD-like receptor signalling pathway were upregulated in most of the treatments, except treatment supplemented with 0.08% *P. amarus* extract. In contrast, striped catfish fed mixture of *P. amarus* and *P. guajava* significantly upregulated proteins related to immune responses including CALR, CD8B, HSP90AA1, HSP90AB1, PDIA3, TUBA1C, CALM1, CCKAR, GRIN2D, VDACZ, ACTB, ACTN4, LCK, CARD9, and VDAC2, whereas they were mostly downregulated or not affected by a single extract administration. To some extent, these results highlighted possible synergistic effects of the mixture supply of *P. amarus* and *P. guajava* extracts on the immune responses in striped catfish liver when compared to the plant extracts alone. Regarding mixture diets, the proteomics results were accompanied with the challenge test. Striped catfish fed 0.08% mixture of *P. amarus* and *P. guajava* could stimulate to upregulate protein related to immune responses and then significantly reduce striped catfish mortality after *E. ictaluri* infection. These positive effects were not observed in the single diets of *P. amarus* or *P. guajava* at the same dose. In addition, the upregulated proteins related to immune responses in the liver were not consistent with the levels of humoral immune parameters (lysozyme, complement, and total Ig) in plasma and mucus after 6 weeks feeding mixture diets. It could be explained that the mode of action of plant extracts was differentially depended on the tissues or organs examined striped catfish.

Furthermore, glutathione plays important roles in antioxidant defense, nutrient metabolism, and regulation of cellular events (Wu *et al.*, 2004). Our study revealed that only treatment

supplemented with the high dose of *P. amarus* diet resulted in the positive effects on the regulation of antioxidation in striped catfish liver (Chapter 6). The results suggested that *P. amarus* greatly responsible for the antioxidant potential, which may be due to the present of high concentration of phenolic compounds (phyllanthin and hypophyllanthin) (Saeed *et al.*, 2012, Simamora *et al.*, 2018, Hamrapurkar *et al.*, 2010, Chirdchupunseree and Pramyothin, 2010). However, the mixture diet at 0.5% did not showed any effects on the antioxidant status of striped catfish liver. It could be suggested that combination between *P. amarus* and *P. guajava* extracts caused the opposite result in antioxidant status. These results together demonstrated that single or mixture supply of *P. amarus* and *P. guajava* extract-based diets positively regulated the immune responses as well as antioxidation of liver proteome profiles, which directly promoted the striped catfish health.

1.5. Mechanism of *P. guajava* extract fractions

As the results above, *P. guajava* extracts potentially improve the striped catfish immune responses. However, the understanding of the mechanism behind the effects of *P. guajava* extracts on fish immune response at molecular level is undoubtedly helpful for proposing appropriate applications in fish farming. In the following chapter (Chapter 8), we isolated the effects of two *P. guajava* extract fractions (i.e. dichloromethane and ethyl acetate) on striped catfish immune responses at different time points (6h, 24h and 72h) after intraperitoneal injection. Our results demonstrated that striped catfish treated with dichloromethane and ethyl acetate from *P. guajava* activated the TLRs-MyD88-NF-κB signaling pathway in response to time and dose of each fraction. Both fractions, especially dichloromethane fraction could promote to upregulation cytokine genes (*tlr1*, *tlr4*, *myd88*, and *traf6*) at the early time-6h. Meanwhile, the cytokine transcripts related to inflammatory (*nfκb*, *tnf*, *il1β* and *il6*), as well as apoptosis (*tp53* and *casp8*) were also increased following the injection. The expression of inflammatory cytokines may contributed to the significant increase of *inos* and *lys* at the later time points-24h or 72h. Similar increase levels of *il1β*, *il8*, *tnfα* and *inos* were observed in rainbow trout macrophages (Na-Phatthalung *et al.*, 2018). Concerning cellular and humoral immune responses, striped catfish treated with both dichloromethane and ethyl acetate fractions did not affect the spleen RBA, spleen NOS and serum lysozyme activities at 6h. The RBA, and NOS levels were inhibited in fish treated with the high dose of dichloromethane fraction. However, these activities were differentially enhanced depending on the dose and type of extract fractions at 72h.

In conclusion, we hypothesize that the activation of the TLRs-MyD88-NF-κB signaling pathway via the enhancement of several cytokines (*tlr1*, *tlr4*, *myd88*, and *traf6*), following the increase of inflammatory cytokines (*nfκb*, *tnf*, *il1β* and *il6*), apoptosis cytokines (*tp53* and *casp8*), lysozyme, iNOS and ROS productions in fish after intraperitoneal administration with *P. guajava* dichloromethane fraction is responsible for the general increase in striped catfish health status.

2. Conclusion

This thesis aimed to investigate the positive capacity of different plant extracts on regulating the immune responses as well as bacterial resistance of striped catfish *Pangasianodon hypophthalmus*. Following the objectives, the functions of plant extracts were examined along with several aspects throughout the *in vitro* and *in vivo* approaches. The results suggested that twenty plant extracts selected from the literature differentially regulated the humoral immune response (lysozyme, complement, and total immunoglobin) in PBMCs and HKLs of striped catfish after 24h stimulation (Chapter 4). Five extracts including *Allium sativum*, *Azadirachta indica*, *Euphorbia hirta*, *Phyllanthus amarus*, and *Zingiber officinale* induced a strong upregulation of 4 cytokines (*il1β*, *ifnγ 2a* and *2b*, and *a2 mhc class II*) according to the concentration, time points and kind of leukocytes (Chapter 4).

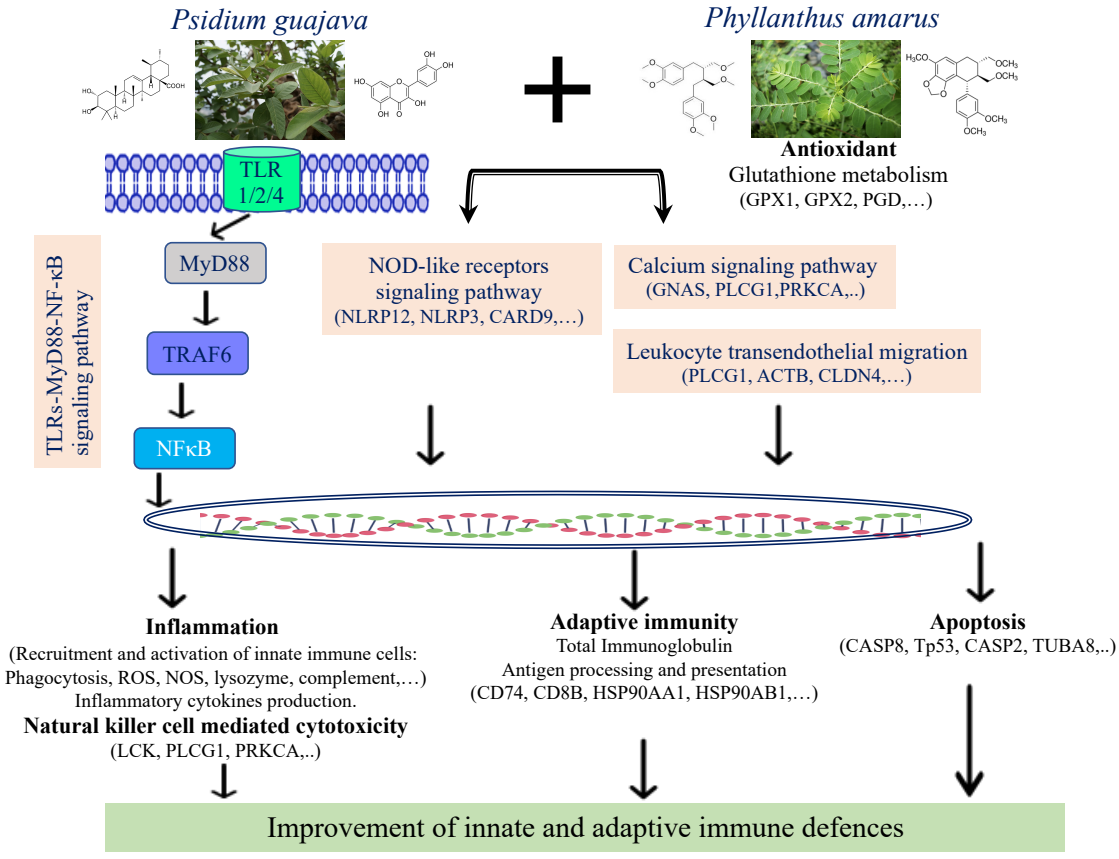

Figure 1. Summarizing the effects of supplemented plant extracts on humoral and cellular immune defenses of striped catfish.

Ethanol extracts of *Phyllanthus amarus*, *Psidium guajava*, *Mimosa pudica*, *Azadirachta indica* and *Euphorbia hirta* could stimulate the striped catfish innate immune response (lysozyme and complement) and adaptive immune response (total Ig) as well as providing better protection of striped catfish infected with *Edwardsiella ictaluri* pathogen (Chapter 5). Moreover, *P. amarus* and *P. guajava* extract-based displayed the better immunoregulation in striped catfish, which also given positive synergistic effects liver proteome profile related to immune system processes when supplying together in the mixture (Chapter 6).

The crude ethanol extracts of *P. guajava* and *P. amarus*, their fractions and the pure compounds at certain concentration can potentially act as immunomodulators in HKLs of striped catfish (Chapter 7). *P. guajava* dichloromethane fraction could enhance TLRs-MyD88-NF-κB signaling pathway, following induce the levels of inflammatory and apoptosis cytokines as well as lysozyme, iNOS and ROS productions, providing the better understanding about the mechanism behind the effects of *P. guajava* extracts on fish immune response at molecular level (Chapter 8). The linking between extracts and immunity is summarized in **Figure 1**.

Taken together, our studies suggested that boosting the immune response by ecologically friendly products, especially *Phyllanthus amarus* and *Psidium guajava* derived products is an effective strategy to promote sustainable aquaculture via improvement the health status as well as resistance to pathogens.

3. Perspectives

Unlike chemotherapeutics, fish were administered with plant products that do not seem to be associated with any side effects. Medicinal plant are also easy to prepare, inexpensive and eco-friendly for environment. Further investigations are needed to isolate, characterize, and quantify the bioactive compounds contained in plant and phytoextracts.

Most of the experiments were undertaken under control conditions, they need to be validated in the farming system, in order to gain global understanding of the effects of plant products on fish health status. Moreover, the comparison of the effects of different parts of medicinal plants, plants collected from different seasons as well as different geographical conditions also need to be investigated.

Further application of plant extract on different sizes of striped catfish in both experimental conditions and on farm will be needed. In practice, a massive mortality of striped catfish is normally happening in 1.0 to 1.5 month and/or 3.5 months after stocking fingerling (50-70 g/fish) which could means that fish have a problem with their immune system at these stages. Therefore, at these stages of culture period could be the key for further studies. Nursing stage of striped catfish would also be interested for further study by applying the active compound for broodstock.

Beside that application period/prolong via administrative feeding would also be another perspective point that further study must pay attention because there is a lack of information on appropriate feeding period of these extracted compound on immune system of fish. Side effects of the compound on immune depression and growth performance that could happen if fish are over-stimulated by these compounds.

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